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Four Wilt Diseases of Perpetual-Flowering Carnations in Denmark

Pseudomonas caryophylli
Pectobacterium parthenii var. *dianthicola*
Phialophora cinerescens
Fusarium spp.

By

ERNST HELLMERS

Contribution No. 51 from the Department of Plant Pathology,
The Royal Veterinary and Agricultural College, Copenhagen.

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Pectobacterium parthenii var. *dianthicola*
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By

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Contents

Preface 9

Introduction 11

I. Bacterial stem-crack of carnation
(*Pseudomonas caryophylli* (BURKHOLDER) STARR & BURKHOLDER) 13
1. Introduction 13
2. Description of the disease 14
3. Isolation of the pathogen 22
4. Infection tests with the isolated bacterium 23
A. Infection tests with rooted cuttings 23
a. Inoculation of stems 23
b. Inoculation of roots 28
B. Infection tests with rootless cuttings 30
a. Introduction 30
b. The anatomy of carnation stems 30
c. Ability of carnation stems to absorb pure water 31
d. Ability of carnations to take up bacteria in aqueous suspension 33
e. Infection tests with *Pseudomonas caryophylli* 34
5. Description of the isolated bacterium 35
A. Morphology and staining reactions 35
B. Cultural characters on different media 37
C. Influence of temperature on growth of the bacterium 38
D. Biochemical reactions 39
6. Dissemination of the disease 41
7. Control 43
A. Direct control measures 43
a. Measures to prevent spreading of the bacteria
during the propagation by cuttings 43
b. Control by means of "Aretan" and 8-hydroxy-quinoline
sulphate 47
c. Control by means of antibiotics 49
i. Testing of the inhibiting effect of various antibiotics
on *Pseudomonas caryophylli* 49
ii. Ability of carnation cuttings to absorb streptomycin, terramycin, Agri-mycin 100, 8-hydroxy-quinoline sulphate, and
Pseudomonas caryophylli 54
iii. Ability of carnation stems to absorb various aqueous
solutions of Agri-mycin 100 in 12, 24, and 48 hours . . . 57

iv. Absorption and distribution of Agri-mycin 100 in stems of carnation cuttings 63
v. Keeping qualities of Agri-mycin 100 in carnation cuttings 65
vi. Reaction of carnation cuttings to treatment with Agri-mycin 100, streptomycin and terramycin 66
vii. Reaction of rooted cuttings to dipping of roots in Agri-mycin 100 68
viii. Conclusions 69
B. Indirect control measures 70
a. Introduction 70
b. The "microscopy method" 71
c. The "agar method" 77
d. The "suspension method" 80
e. The "broth method" 82

II. Bacterial stunt of carnation (*Pectobacterium parthenii* var. *dianthicola* var. n.) 95
1. Introduction 95
2. Description of the disease 98
3. Isolation of the pathogen 105
4. Infection tests with the isolated bacterium 106
A. Infection tests with rooted cuttings 106
a. Inoculation of stems 106
b. Inoculation of roots 108
B. Inoculation of rootless cuttings 109
5. Description of the isolated bacterium 110
A. Morphology and staining reactions 110
B. Cultural characters on different media 111
C. Influence of temperature on growth of the bacterium 113
D. Biochemical reactions 114
E. Comparative infection tests with carnation isolates and isolates of *Erwinia carotovora*, *E. atroseptica*, *E. chrysanthemi*, and *E. carotovora* f. sp. *parthenii* 116
6. Discussion of the taxonomy and nomenclature of the isolated bacterium 117
7. Dissemination of the disease 131
8. Control 132
A. Direct control measures 132
B. Indirect control measures 133

III. The incidence of the carnation bacterioses—as well as of *Phialophora cinerescens*, *Fusarium dianthi*, and *Fusarium culmorum*—in Denmark . . 141
1. Introduction 141
2. Material and methods 141
A. The plant material for the investigation 141
B. Investigation technique 142
3. Results of the investigation 144
A. Cause of the disease: Bacteriosis 144
B. Cause of the disease: Mycosis 148
C. Cause of the disease: Non-parasitic factors 149

IV. Appendix. Wilt disease in Danish perpetual-flowering carnations, caused by parasitic fungi 150
1. Fan-mould of carnation (*Phialophora cinerescens* (Wollenweber) van Beyma) 150
A. Introduction 150
B. Description of the disease 153
C. Control 157
2. Collar-fusariosis of carnation (*Fusarium dianthi* Prillieux & Delacroix, *Fusarium culmorum* (W. G. Smith) Saccardo, and other species of *Fusarium*) 162
A. Introduction 162
B. Description of the disease 163
C. Control 165

V. Review of differentiation of the four carnation wilt diseases described . . 167

Summary 171

Literature 184

Dansk Resumé 188

Preface

The investigations reported upon in this paper were carried out, in the main, at the Department of Plant Pathology of The Royal Veterinary & Agricultural College, Copenhagen, Denmark, in the years 1953–56. Professor N. Fabritius Buchwald, the head of the Department, allowed me free conditions for carrying out this work and, furthermore, rendered me valuable help in many ways, for instance in the matter of nomenclature, and I wish to thank Professor Fabritius Buchwald most cordially for his kind assistance.

Many of the investigations were made in the nurseries of Mr. Knud Stormly-Hansen, Vanlöse, and Mr. Aage Frandsen, Avedöre, both of whom placed plants, greenhouses and manual help at my disposal with the greatest readiness, even for large and costly experiments. I wish to thank both of them sincerely for the great assistance they gave me in this way. Not least am I grateful to these two gentlemen for their pertinent criticism, for their permission to avail myself freely of their extensive horticultural knowledge, and for frequent consultations on the growing of carnations.

The funds required for carrying out the investigations were provided by grants from The Technical-Scientific Research Council, The Danish State Science Fund, The Horticultural Research Foundation, the firms of H. A. Möller, and Leo Pharmaceutical Products Trading Ltd., Copenhagen, Denmark. I welcome this opportunity of tendering these foundations and firms my heartiest thanks for their financial aid, without which it would have been impossible for me to do this work.

I acknowledge with particular gratitude the kindness of my teacher, W. J. Dowson, D.Sc., Lecturer in Mycology and Plant Pathology at the Botany School, University of Cambridge, England, with whom I studied plant pathological bacteriology in 1947, and who has since followed my work with the greatest interest, rendering me extremely valuable support and guidance. Moreover, Dr. Dowson has corrected

the English of the manuscripts of this paper as well as others of my papers, for which I am most grateful to him.

The two electron micrographs in this paper were taken by A. Birch-Andersen, M.Sc., of Statens Seruminstitut, Copenhagen, Denmark; it was a very wearisome job, and also very costly, but the Institute has generously placed the photographs at the author's disposal free of charge. I wish to express my heartiest thanks to the Institute and the staff of its Department for Electron Microscopy for the valuable assistance I have had. All other photographs have been taken by the author.

Furthermore, I wish to extend to Professor Erik J. Petersen and Horticultural Adviser Asger Klougart my best thanks for much good advice during my work.

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Copenhagen, October 1957.

Ernst Hellmers

Introduction

According to concordant statements of elderly carnation growers "wilt disease of carnation"—or "svindsot" as it is also called in Denmark—has been known in this country at least since 1915–20, that is, right back to the time when more intensive cultivation of the so-called American remontant carnations was begun.

Sudden withering of entire carnation plants may, however, be due to several different causes: excessive watering, drought, and various chemicals, for example, may harm the very sensitive root system to such an extent that the plants wither up completely.

Apart from the abiotic causes, "wilt disease" may arise in consequence of attack by pests (nematodes, gnawing insects, etc.), parasitic fungi and bacteria, but the chief cause of what is generally understood by "wilt disease of carnation" is attack by fungi and bacteria.

Although "wilt disease" has from early times given rise to much trouble in the cultivation of carnations and is still inflicting very heavy losses on Danish growers, no really thorough analysis of the etiology of this serious disease has been made so far in Denmark. Nor were the present investigations of "wilt disease" in Danish perpetual-flowering carnations originally started as a general examination of the various causes of the "wilt disease"; they were commenced in the autumn of 1953 as a bacteriological examination of a bacterial disease which caused extensive withering of large numbers of carnation cuttings. In this examination, *Pseudomonas caryophylli* (BURKH.) STARR & BURKH. was demonstrated as the prime cause of the disease, and effective measures for the control of this bacteriosis, which was new to Denmark, were elaborated.

While the exploration of the aforesaid bacteriosis was going on, the author found a species of bacterium hitherto undescribed, which, like *Pseudomonas caryophylli*, was able to produce a wilt disease in carnations. This new bacterium was found closely related to *Erwinia chrysanthemi* BURKH. et al., and it was demonstrated practically simultaneously both

in Holland (BAKKER & SCHOLTEN 1955) and England (LELLIOTT 1956), from where preliminary descriptions of the new carnation pathogen are issued. The author has also subjected this species of bacterium, *Pectobacterium parthenii* var. *dianthicola* var. nov., to a closer examination in respect of its pathogenicity, taxonomy and the control of the disease.

When, in May–June of 1956, the work with the two bacterioses was nearing its completion, the author had occasion to examine some withering carnations, partly from a Swedish nursery and partly from three Danish nurseries, and thereby demonstrated attacks of the fungus *Phialophora cinerescens* (WOLLENWEBER) VAN BEYMA in all the plants examined. Especially in Holland and England, this fungus is a seriously dreaded carnation pathogen, which had not so far been recorded for Denmark. The author, then, deemed it the most proper thing to terminate the bacteriological examinations by a general pathological analysis of carnations suffering from "wilt disease" in all Danish carnation nurseries, partly for the purpose of establishing the incidence of the two bacterial diseases in Danish carnation crops, partly in order to find out how great a part *Phialophora cinerescens*, and possibly other fungi, such as *Fusarium* spp. was playing as a cause of "wilt disease". These examinations—which were carried out in June–July of 1956—have been incorporated in this paper as an appendix.

Chapter I

Bacterial Stem-Crack of Carnation

(*Pseudomonas caryophylli* (BURKHOLDER) STARR & BURKHOLDER)

1. Introduction

In October 1953 The Department of Plant Pathology of The Royal Veterinary and Agricultural College, Copenhagen, received from a Copenhagen nursery a few hundred carnation cuttings of the variety "Harvest Moon". The plants received were just fit for planting-out or sale. They were some five weeks old, about 15–20 cm high and had a well-developed root system. The cuttings were of a greenish-blue colour and, at first glance, showed no outward sign of disease. It was stated, however, that several of these young plants wilted after a short time, whether transplanted into pots or into raised beds in greenhouses.

The cuttings were immediately subjected to a preliminary examination, which disclosed a very faint brownish discoloration of the vascular tissues in many of the stems. In cross sections of the stems the discoloured tissues appeared as brown spots in the xylem, and in longitudinal sections they were most frequently seen as long, narrow or broad stripes coming out from the stem base and extending from about 1 cm to 5–8 cm up the stems.

Examination under the microscope of the discoloured tissue in the border zone between healthy and diseased tissues failed to disclose the presence of fungal hyphae, but a great number of bacteria were constantly observed. In addition to this, a great deal of the vascular tissue appeared to be decomposed, and the resulting cavities were filled with a brownish-yellow bacterial slime.

Everything thus seemed to indicate that the carnations were attacked by a vascular bacteriosis, and it was therefore decided to isolate the bacteria observed in the diseased vascular tissue. For safety's sake, isolations were also carried out with a view to any parasitic fungi, but

in all isolation tests, both on Difco potato dextrose agar (PDA) and nutrient agar, the same type of bacterial colony was found, which, after 6 days, appeared on PDA as a chocolate-brown, pulvinate, entire-margined colony, and it was so tough that most of the colony stuck to the inoculation needle when transfer was made.

Bacteriological examination and infection tests with an American isolate[1]) and with the Danish ones proved with certainty that the species of bacterium isolated from the carnation variety "Harvest Moon" is identical with *Pseudomonas caryophylli* (BURKHOLDER) STARR & BURKHOLDER (1942).

This bacterium produces a disease in perpetual-flowering carnations, in America known as "bacterial wilt of carnation". Since the disease was first described, a similar bacteriosis ("wilt disease") produced by a species of *Pectobacterium*, has, however, been observed in carnations in Denmark, England (LELLIOTT 1956) and Holland (BAKKER & SCHOLTEN 1955), and it is therefore inappropriate to use the term "wilt" in the designation of either of these diseases, this term being already applied to denote attacks of both *Fusarium* and *Phialophora*. The author therefore suggests that the carnation bacteriosis caused by *Pseudomonas caryophylli* be called "bacterial stem-crack of carnation" (in Danish: "Nellikens Sprækkebakteriose"), because a conspicuous cracking of the stem, particularly on younger carnations, has proved to be a characteristic symptom of this bacteriosis. For the *Pectobacterium* species, see Chapter II, p. 95.

2. Description of the Disease

History and distribution. The literature available on the bacterial stem-crack of carnation is very scant. The disease was first observed in perpetual-flowering carnations of the varieties "Kathryn", "Melrose" and "King Cardinal" in 1940 in Spokane (Washington), U.S.A., and was briefly mentioned the following year by L. K. JONES (1941). JONES described the disease as a wilt disease resembling very much the well-known wilting caused by *Fusarium*. He further stated that the new disease produced characteristic yellow stripes, up to 12–24 inches long, of bruised cells in the xylem of the stem. Curiously enough, JONES does not mention any formation of cracks in the stems. From the diseased tissues, bacteria were isolated which, when inoculated into the stems of healthy carnations, were able to produce, in a month's time, the same

---

[1]) Received from W. J. DOWSON's collection of isolates.

newly observed "bacterial wilt". JONES further mentions that 5, 15 and 30 per cent., respectively, of the varieties "King Cardinal", "Melrose" and "Kathryn" were found to be killed by the bacteriosis, and that cuttings from plants which had not yet displayed symptoms of "wilt" were able to transmit the bacteria. As these were different to any of the plant pathogenic bacteria previously described, the isolates were sent to Professor W. H. BURKHOLDER at the Cornell University (in 1940) for further examination. Already in February 1942 BURKHOLDER gave a detailed description of the bacterium and established it as a new species under the name of *Phytomonas caryophylli* sp.n., which M. P. STARR—in collaboration with BURKHOLDER—changed 4 months later, viz. in July of the same year, into *Pseudomonas caryophylli* (BURKHOLDER) STARR & BURKHOLDER (1942).

Since then, bacterial stem-crack of carnation has been found in several places in the U.S.A. (GREGORY 1942, TILFORD & ALGER 1947), where it has been mentioned frequently in special flower and carnation journals (Florist's Review (CREAGER 1942), Carnation Craft (GUBA 1952), etc.), but it does not yet seem to be reported from other countries. It should be observed, however, that *Pseudomonas caryophylli* has been reported from Holland by M. BAKKER & G. SCHOLTEN (1955), but during the 14th International Horticultural Congress in Holland in September 1955 the author of this paper demonstrated that the determination was wrong, and that the bacterium from the Dutch carnations was not identical with *Ps. caryophylli*, but with the already mentioned new carnation pathogen, which may be referred to the genus *Pectobacterium* WALDEE (1945) cf. Chapter II, p. 95.

While working on the identification of the disease in Danish carnations the author has, furthermore, found *Ps. caryophylli* in cuttings imported from Sweden, Germany and Holland. From Germany also M. STAHL (1955) reports on the possible occurrence of *Ps. caryophylli*. In England the author has found specimens of bacterial stem-crack in a single nursery in 1954 among cuttings of the variety "Dark Red Sim", imported from Denmark, but the whole lot was immediately burnt with soil and pots.

As far as is known, bacterial stem-crack of carnation has never before been observed in Denmark. For several years it has been believed that the wilt, which, in practice, is called "wilt disease of carnation", is caused not only by species of *Fusarium*—which have been observed in wilted carnations, e. g. by The Danish Plant Pathological Research Station (1922, 1931, and 1932)—but that there must also be "something

else" wrong with the carnations. The "wilt disease" has, however, not previously been thoroughly investigated in this country, and it is thus impossible to say for certain whether the disease in Denmark has all the time been caused by one or more parasitic fungi (*Fusarium*, *Phialophora*[1]), etc.), or whether bacterial stem-crack may also have contributed to this supposed complex of symptoms. The symptom common to the diseases mentioned—the wilting—might very easily lead to confusion, but it is likely that bacterial stem-crack of carnation is a comparatively new disease in Danish perpetual-flowering carnations. Otherwise, the bacteriosis would, to all appearances, have made itself felt earlier in the great number of intensely propagated cuttings which have been produced in this country; in fact, the malignant stem cracks in young carnations do not seem to have been observed before. As far as the author is able to judge, the first appearance of bacterial stem-crack in Denmark dates back only to the period 1946–51 when, after the end of World War II, the imports of new American varieties of carnations increased considerably (KLOUGART 1956), and presumably the disease has been introduced with cuttings of these novelties. The introduction of the bacteriosis need not, however, have taken place directly from America. It may very well have taken place via other countries having a lively exchange of cuttings with both America and Denmark. At any rate, after the war the bacteriosis has gradually been increasing in some Danish nurseries until, about 1950–51, it began to make itself felt seriously and in 1952–53 it had become catastrophic in a few nurseries. Among the lots of cuttings of the varieties "Johanna", "Dark Red Sim" and "Harvest Moon" which were examined immediately in the autumn of 1953 it was usual to find 40–60 % diseased plants; in a single case even 75 % plants attacked by bacteria were found in the variety "Harvest Moon".

Symptoms and etiology. Bacterial stem-crack of carnation is a typical vascular bacteriosis, attacking solely the vessels in the stem and roots of the plant (Figs. 1 & 2).

In serious cases the result is that the carnation plants soon wither, assuming a non-characteristic greenish-grey discoloration, but the disease may also take an insidious course, during which deep longitudinal cracks

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[1]) Attacks of *Phialophora cinerescens* are often mentioned by Danish carnation growers but up to 1956 the fungus had not been demonstrated in Denmark! While working with the bacteriosis the author has, however, in several cases managed to identify attacks of *Phialophora cinerescens* in Danish carnations and to isolate the fungus (see also Chapter IV, p. 150).

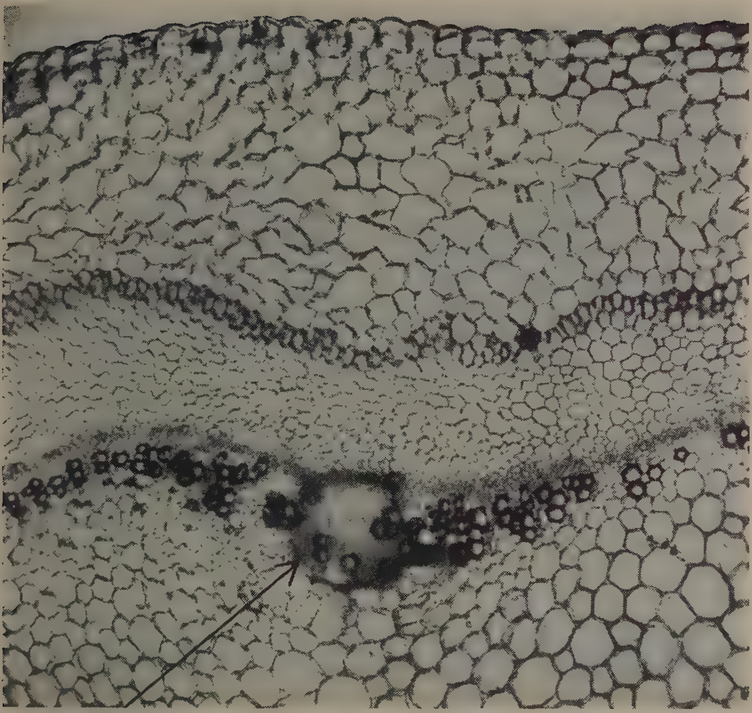

Fig. 1. Cross section of stem of "Harvest Moon", naturally infected by *Pseudomonas caryophylli*. The infection (at the arrow) involves only about a dozen vessels. Photographed 8.12.1953. ×125.

are formed in the lower internodes of the stems in young plants (Fig. 3). The bacteriosis may even develop so slowly that slightly infected plants will survive the usual 2–3 years' cultivation period without any immediate, visible signs of the disease. In the course of time, cuttings whose vessels are attacked by bacteria may inadvertently be taken from such plants.

Thus, bacterial stem-crack is transferred with the infected cuttings taken from diseased mother plants, and it might then be expected that the first, faint signs of the disease could be seen when the cuttings were subjected to a closer examination, but it should be emphasized here, that in cuttings newly broken off the disease is much retarded. The only thing which can be observed in some cases is the occurrence of brownish-

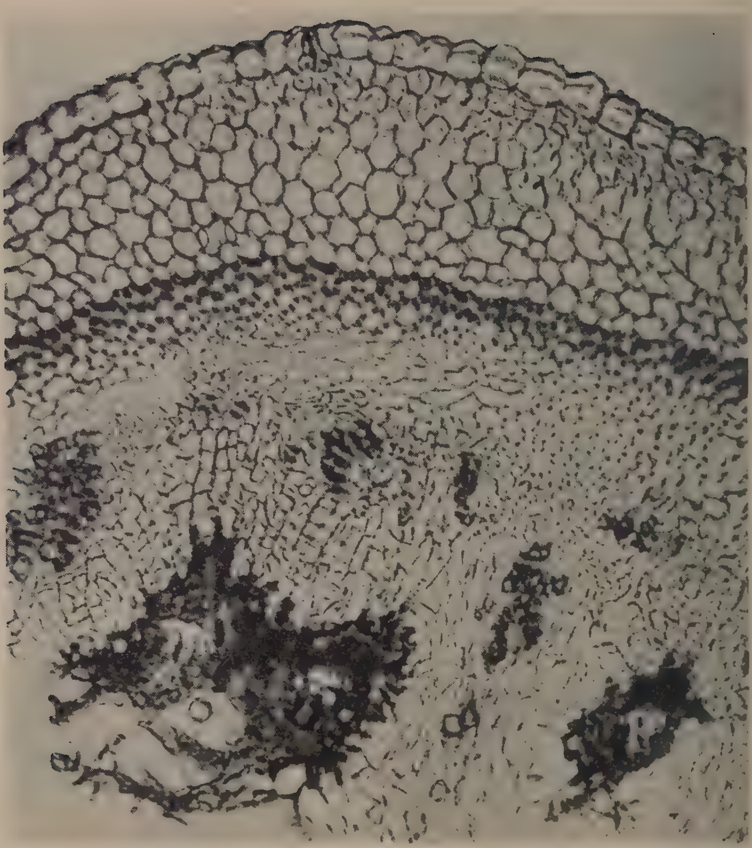

Fig. 2. Cross section of stem of "Johanna", attacked by *Pseudomonas caryophylli*. Note, at the bottom of the picture, the two large necroses and the formation of new vessels in the new callus cells in replacement of the vessels destroyed by the bacteria. Some of the new vessels are also attacked. ×150.

yellow spots in the vascular tissue in a cross section of the stem, originating from attacked vessels, but very slight attacks cannot be ascertained even with the aid of a microscope.

When such primarily infected cuttings are placed in the sand of the propagation bed, the bacteria make their way from the infected vessels into the water of the propagation bed and are taken up by adjacent healthy cuttings, which are thereby infected in a greater or lesser degree. Whether the cuttings have adopted the disease in the propagation bed or have carried it along right from the mother plants, the heavily infected

Fig. 3. Young carnation plant of the variety "Petersen's Pink Sim", artificially infected with *Pseudomonas caryophylli*, isolate No. 83. Photographed one month after inoculation (inoculation done at the dark spot (at the arrow) near the lowermost crack). Note the deep cracks. Natural size.

shoots will wither without forming roots and without displaying symptoms of the disease deviating from normal withering caused, for instance, by drought. On the other hand, the more slightly infected cuttings are able to form some roots and thus grow to some extent, whereby they

come to resemble the healthy cuttings so much that the disease cannot be recognized on a cursory inspection at this stage of the life of the carnation.

Things look different, however, when, after the usual 4 or 5 weeks' stay on the propagation bed, the cuttings have formed roots and are ready for transplanting into beds. From this stage the diagnosing of the bacterial stem-crack will be most reliable. The heavily infected young plant will not stand being transplanted and will soon die. Prior to this sudden withering the uppermost leaves of these plants can often be seen to be a little paler—with a greyish hue—than those of healthy carnations; nor have the leaves the curl so characteristic of healthy carnations. On the other hand, it may happen that the pale, greenish-grey top leaves are twisted abnormally before withering (Fig. 4).

If only slightly infected, the young plants will for a long time have a normal growth, at the same time as they try to seal off and replace the diseased vascular tissue by a vigorous internal callus formation, while, on the other hand, the bacteria destroy the vessels. This struggle between plant and parasite, which usually ends with the victory of the latter, may be short-lived, particularly if the temperature in the greenhouse is high, i. e. upwards of 20–25°C, but under cooler conditions it may drag on for weeks or months, or even years. The rapid formation of meristematic cells in the stems generally leads to a tension in the tissues around the vessels (Figs. 5 & 6, pp. 25 & 26), which again results in bursting of the cortex of young plants, whereby the above-mentioned longitudinal cracks occur in the lower internodes (Fig. 3). In the cracks a brownish-yellow bacterial slime is seen which, in time, is often overgrown with saprophytic fungi such as *Cladosporium herbarum*, etc., so that eventually the wounds may assume a piebald, unsavoury appearance (Fig. 21, p. 103). The formation of these more or less deep, longitudinal cracks may finally induce such serious disturbances to the vascular tissue of the plant that the whole of the plant wilts, assuming a not particularly characteristic greenish-grey discoloration. While the formation of cracks is going on, the root system of the plant will generally be relatively well preserved, but when the wilting occurs it is usually more or less decomposed, so that the plant may easily be pulled out of the soil.

Plants with large stem cracks may, however, also develop shoots of a normal appearance (Fig. 21, p. 103), so that a closer examination of the stem is required to find the diseased plants among the healthy ones, but frequently the healthy looking shoots will contain vessels filled with bacteria. Finally, the plants may happen to be growing very fast, besides

Fig. 4. Right: Top shoot of a two-month-old carnation plant of the variety "Harvest Moon", naturally infected by *Pseudomonas caryophylli*. Note the twisted primordinal leaves. Left: Top shoot of a healthy plant of the same lot. ×0.4.

being so slightly infected that no cracks and no wilting occur before the ordinary termination of the crop after 2 or 3 years. In such older carnation plants the bacteriosis does not seem to have a pronounced tendency to cause formation of cracks in the stems, which fact may be due to their more woody structure. In many slightly infected plants it is therefore difficult—not to say impossible—to recognize the disease without laboratory testing, but since, after all, the bacteriosis exists in a smaller or greater number of vascular tissues, these plants may easily act as "healthy" carriers of infection, thus constituting the origin of infected cuttings.

The destruction of the vascular tissues of the carnations by the bacteria will, however, also cause many older plants to die. Assuming its normal greenish-grey colour this wilt does not differ essentially in appearance from other "wilt diseases" of carnations caused by bacteria as well as fungi. A closer examination of the withered plants will, however, disclose

that, in the first place, they are very easily pulled out of the soil,—indeed, in certain cases they have hardly any connection with the soil because their roots are entirely decomposed; secondly, if the bark is peeled off into the vascular tissue, brownish-yellow, narrow or broad, longitudinal stripes are seen; in cross sections these stripes appear as irregular, brownish spots with a water-soaked margin (Fig. 39, p. 168). The discoloured parts of the vascular tissues are also very sticky, which is a very characteristic feature in older carnations attacked by bacterial stem-crack, and in this way the disease is definitely distinguishable from the "wilt diseases" caused by other bacteria or fungi.

From the foregoing description it will appear that bacterial stem-crack of carnation does not present a single external, easily recognizable symptom. Neither formation of cracks, nor wilting, nor decomposed roots, nor sticky vascular tissues are symptoms which are always apparent in attacked plants. To be able to form a reliable diagnosis it is necessary to examine many plants, both young and old, and to cut open the stems and—which is highly preferable—to make isolates from the diseased parts of the stems.

3. Isolation of the Pathogen

Since, after examination of the carnation cuttings received, it was evident that it was particularly the large content of bacteria in the discoloured vascular tissue that might be considered to be the cause of the poor growth of the carnation plants after the planting-out, isolation from the bacteria-filled vessels was attempted. Some ten plants were selected, whose entire root system was cut off together with about 1 cm of the stem. The cut-surfaces thus produced on the stems were then examined through a hand lens, and some plants with a distinctly brownish-yellow discoloration of the vascular tissue were selected. From these plants five plants again were selected, whose lowermost internodes were thoroughly rinsed in 70 % ethanol, after which the cortex of the stems was cautiously peeled off with a flamed scalpel (Gillette surgical blade, shape E, No. 3). In this way the discoloured vascular tissue could fairly easily be exposed, and after the border zone between the upper portion of the discoloured vascular tissue and the healthy tissue had been examined, small pieces (3 from each of the 5 plants) of the discoloured vascular tissues were removed with a sterile Gillette scalpel. One of the three pieces from each cutting was placed directly on potato dextrose agar (PDA), and one on nutrient agar in Petri dishes, while the third

was transferred to 1–2 ml sterile, distilled water in a test tube. After a few hours, numerous bacteria were found in the water in all five tubes, after which the suspensions were streaked on to Petri dishes containing PDA and nutrient agar (Difco), respectively. For each suspension, two dishes of each medium were used, i. e. 20 dishes in all. All the dishes were then incubated at 27°C, and after two or three days a great number of uniform bacterial colonies appeared around the pieces of vascular tissue as well as on the surfaces of agar on which the suspensions had been streaked, but in no case did fungal hyphae appear.

The bacterial colonies on nutrient agar have a yellowish appearance—almost like that of *Xanthomonas*. On PDA whitish-grey, entire-margined, pulvinate colonies were formed at first; after about 6 days at 27°C they turned light brown and later on dark chocolate-brown with a narrow, pale, cream-coloured margin. This change in colour did not occur on ordinary nutrient agar, but the addition of $0.5^{0}/_{0}$–$1^{0}/_{0}$ glucose made the colonies assume an even deeper dark-brown colour than on PDA. Incidentally, the colonies became so tough after 5 or 6 days that most of the colony stuck to the inoculation needle when transfer was made.

As in all the isolations only one and the same type of bacterial colony appeared, and as the space available in greenhouse and laboratory did not permit working with several isolates, a single isolate—No. 83—was chosen at random for infection tests and further bacteriological investigations.

4. Infection Tests With the Isolated Bacterium

A. Infection Tests With Rooted Cuttings

a. Inoculation of Stems

To investigate whether isolate No. 83 was able to produce bacterial stem-crack in healthy carnations, a small-scale preliminary infection test was made in the first place, for which about 6-week-old plants of the variety "Harvest Moon" were selected from among an apparently healthy lot. Of the plants selected, 10 were inoculated with bacterial slime from a 4-day-old PDA culture of isolate No. 83. Another 10 plants from the same lot were inoculated with sterile, distilled water as checks.

The inoculation was done in the following way: into one of the lowermost internodes of the stem of each test plant three tiny closely spaced holes were pierced, into which bacterial slime from isolate No. 83 was smeared immediately by means of a flamed inoculation needle. The

check plants were inoculated only with sterile, distilled water. Incidentally, the holes were made with a very sharp-pointed, flamed needle and were roughly made so deep that the point of the needle just passed beyond the xylem.

After the inoculation, the plants were left directly on the benches in a relatively light greenhouse at a temperature of about 16–20°C, and had normal care and attention during the following months. During the first two or three weeks no sign of disease was seen in the young carnation plants, but after exactly one month it was observed that in all the plants that had been inoculated with bacterial slime, the internodes began cracking just above the point of inoculation, and a yellowish, sticky bacterial slime could be seen in each crack. In the following weeks the cracks became broader and longer—frequently almost as long as the internodes—, and similar cracks appeared in several of the internodes higher up the stems (Fig. 3).

Gradually as the cracks widened, they appeared not to pass radially into the stems, but after the epidermis had burst they described almost a chord to the periphery of the cross section of the stem. This apparently strange condition, which was also met with in naturally infected plants, was investigated more closely by the making of cross sections at several points up the stems, in the hope of finding out how the cracks were formed.

From the transverse sections of the infected carnation stems it seems that—possibly through the production of some growth promoting substance—the bacteria are able to stimulate the cells around the attacked vessels to a vigorous production of meristematic cells (callus formation) (Fig. 5). If several vessels are attacked (Fig. 6), the formation of callus around the diseased vascular tissue becomes so violent that the surrounding tissue—particularly where the diseased vessels border upon healthy ones—is unable to keep pace with the abnormal development of cells and therefore bursts (Figs. 5 & 6). Thereby cavities may arise in the stems, which the plants try to fill out by the formation of callus from the edges of the torn cells (Figs. 5 & 6). The vigorous callus formation inside the stems may eventually have the effect that the epidermis is also burst (Fig. 3, Fig. 21, p. 103), after which the aforementioned stem-cracks occur. The source of the crack across the longitudinal direction of the stem follows the ring of attacked vascular tissue. Accordingly, the formation of cracks takes place by cleavage of the diseased vascular tissue or, in other words, by peeling-off of cortex parallel to the periphery of the cross section.

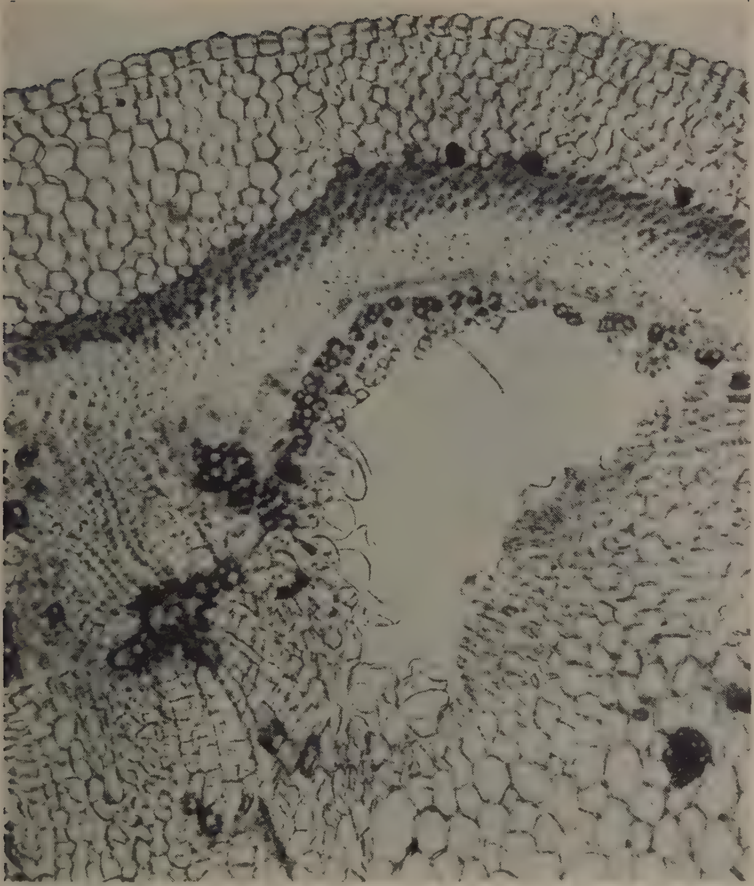

Fig. 5. Cross-section of stem of "Harvest Moon", naturally infected by *Pseudomonas caryophylli.* The attack is somewhat more extensive than that shown in Figs. 1 & 2, and the plant has formed so many new cells around the attacked vessels that a fissure has formed between the vascular tissue and the pith. On the outside of the plant nothing is yet visible. Note also the large callus cells at the edge of the fissure. ×150.

It was remarkable how little these deep stem-wounds affected the growth of the plants. Even after 3 months, the top shoots were practically normal, although the stems were so full of cracks that it was hard to believe that the plants could survive with so heavily damaged stems (Fig. 21, p. 103). All the plants inoculated with bacteria wilted, however,

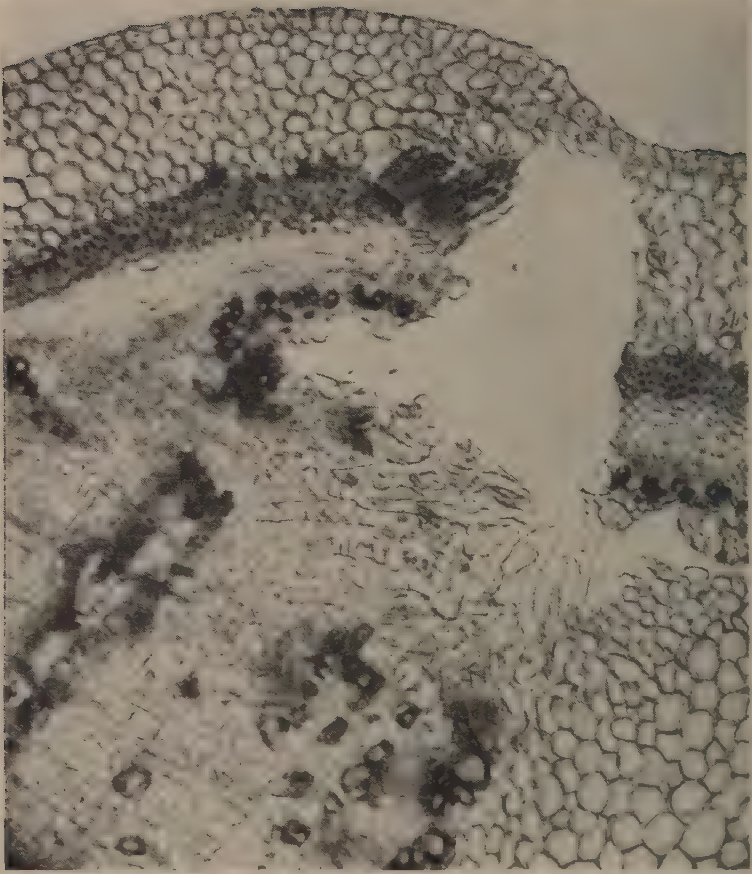

Fig. 6. Similar to Fig. 5. The destruction of the vascular tissue has further advanced, and the bursting of the epidermis is impending. Note also the voluminous callus cells at the edge of the fissure. ×150.

after 6–8 months. All the check plants stayed healthy, and in these plants the inoculation wounds healed in the normal way.

As soon as the top shoots of the infected plants had wilted, the interior stem tissue was examined under the microscope, whereby it was possible to see that several vessels were filled with bacterial slime. Some had even disintegrated entirely, and, altogether, the bacteriosis had spread high up in the plant. In some of the plants a few brown vessels could be traced right up to the top shoots. On the other hand, the disease seemed not—

or but slightly—to have penetrated to the roots; most of the test plants had a healthy and intact root system.

From the discoloured vascular tissue isolation was made by cutting out, aseptically, small pieces, which were transferred to sterile, distilled water, some of which was streaked on to PDA in Petri dishes after a few hours. By this re-isolation the characteristic bacterial colonies of *Pseudomonas caryophylli*, at first greyish, later on dark-brown, were produced in pure culture, and thus there could no longer be any doubt about the pathogenicity of the original isolate (No. 83). From the dishes with the re-isolate, another isolate (No. 84) was again selected at random, and after re-inoculation in young carnation plants of the variety "Harvest Moon" it produced stem-cracks in the lowermost internodes after one month.

As the infection method described above had proved to be fully applicable, it was decided to carry out an infection test on a larger scale with a view to investigating the pathogenicity of the two isolates (Nos. 83 & 84) towards some of the other carnation varieties commonly grown. For this purpose, test plants of the following 8 varieties were obtained: "Dark Red Sim", "Peppermint Sim", "Petersen's Pink Sim", "Salmon Sim", "White Sim" and "William Sim", all of which belong to the so-called "Sim" varieties, as well as of the varieties "Johanna" and "Sidney Littlefield", which are not members of the "Sim" family.

The test plants, which were chosen from lots of carnations that had hitherto proved to be healthy, were from 5 to 7 weeks old (in 3-inch-pots), about 20 to 30 cm high, and not yet pinched. Altogether 15 plants of each variety were used: 5 for inoculation with isolate No. 83, 5 for inoculation with isolate No. 84, and 5 for inoculation with sterile, distilled water as checks. After inoculation, all the test plants were left on benches in a greenhouse at a temperature of 18–25°C. Again it took exactly one month before the lowermost internodes on the plants inoculated with bacteria began cracking as described above. The check plants stayed healthy throughout the four months the test lasted. The pathological picture was remarkably uniform in all the carnation varieties used, and it was thus not possible to ascertain any difference in resistance.

Nor do the "Sim" varieties seem to be more susceptible than other varieties—in this case "Johanna" and "Sidney Littlefield"—, which was also borne out by the fact that the varieties in which the bacteriosis was first observed, viz. "Kathryn", "Melrose" and "King Cardinal", cannot be referred to the "Sim" varieties either. Incidentally, this applies also to the following thirteen American varieties, which Jones and Burkholder

found susceptible by artificial infection: "Chief Kokomo", "Giant Laddie", "Joan Marie", "King Cardinal", "Matchless", "Maytime", "My Love", "Patrician", "Pink Spectrum", "Puritan", "Sophelia", "Virginia", and "White My Love".

b. Inoculation of Roots

As far as the author knows, no literature is available on experiments with, or observations of, the ability of the bacteria to live in soil, and so infecting the carnation plants through this channel. The question is, however, only of limited importance to carnation cultures, seeing that with a view to avoiding fungus diseases it has gradually been found absolutely necessary either to replace the soil from the carnation beds entirely with completely fresh field soil which has never been in contact with carnations, or to sterilize the used soil very thoroughly—usually by steam—after the termination of each culture,—indeed, some growers may go so far as to sterilize even the fresh field soil to avoid Nematodes. Transfer of diseases from one culture to another by the application of soil which has previously been in contact with carnations is very improbable today, or at any rate not compatible with good methods of carnation growing. On the other hand, whether caused by fungi or bacteria, the various diseases seem to be highly transferable from one culture to another, because the organisms are capable of keeping alive in cavities in the cement slabs, boards and other bench material—often even in spite of thorough cleaning with soapy water, lime-washing, etc. As far as bacterial stem-crack is concerned, the author observed, in the spring of 1954, three small localities ("spots") with cuttings that had wilted in consequence of bacterial stem-crack on a certain propagation bench in a Copenhagen carnation nursery. The location of the "spots" in the greenhouse was, by sheer accident, marked off on the window bars of the greenhouse, and on termination of the culture the whole bench was cleaned very thoroughly, all the boards being washed with soapy water and lime. Next, the gravel was renewed completely, after which a batch of newly potted cuttings (in new pots) were placed on the bench. When the roots of these plants had grown through the pots and the sand on to the boards, attacks of bacterial stem-crack were observed in exactly the same three places as in the previous crop. As there had been no infected plants in the lot in question, the bacteria must in this case be assumed to have been transferred from one crop to the other through the bacteria-carrying boards, and as similar "cases" later on

were observed in other carnation crops, the bacteria must be assumed to be able to keep alive at any rate for a few weeks in old, damp boards.

In further support of this theory, a small-scale infection test was made with 30 young carnation plants of the variety "White Sim". After their root systems had been shortened somewhat with a pair of scissors, 10 of these plants were potted up with soil in which carnations attacked by bacterial stem-crack had grown previously and which thus contained a good deal of decomposed roots from these diseased plants; 10 plants were potted in sterile pots with autoclaved soil; the remaining 10 plants were also potted in sterile pots and soil after having been dipped in a suspension of isolate No. 83. After potting, all the plants were left on benches in a greenhouse at a temperature of 18–25°C, and only after 4 to 6 weeks did the plants which had been dipped in the bacterial suspension begin to wilt—though without any formation of cracks, but with a heavily decomposed root system.

Of the 10 plants which were potted in infested soil, 6 wilted after 2 months. Also in this case did the wilting take place without formation of cracks on the stems—with a single exception, however. This may presumably be explained by the fact that in both tests the inoculation has been so heavy that the bacteria had damaged the root systems through the wounded roots, so much that the growth of the plant—and consequently the formation of cracks—has been stopped entirely. The roots of all the withered plants were completely decomposed after the end of the test period, and bacteria-filled vessels could be traced to the middle of the stems.

The 10 plants in the autoclaved soil (the check plants) remained healthy throughout the two months the test lasted, after which their root systems were wounded by the insertion into the soil—midway between the stem and the edge of the pot—of a new, about 2 cm wide, garden label. After removal of the label, the holes thus produced in the soil of 5 plants were filled up with a suspension of *Pseudomonas caryophylli*, isolate No. 83. The remaining 5 plants were watered with ordinary tap-water. All 10 plants were again left for observation, which was terminated after 2 months, with the result that 4 of the inoculated plants had wilted without formation of cracks, while the rest of the plants had stayed healthy.

The abovementioned tests thus seem to bear out the assumptions that carnation plants with wounded roots can be infected through the root system and that *Ps. caryophylli* is able to live in the soil for some time, the latter of which was further confirmed by the fact that the organism

keeps well for at least one year in autoclaved, moist garden soil at 10°C (cf. p. 38). When the infection takes place in this way and is so violent as in the abovementioned infection tests, the plants are apparently damaged to such a degree that their growth, and consequently the formation of stem cracks, is prevented.

B. Infection Tests With Rootless Cuttings

a. Introduction

During his work with bacterial stem-crack of carnation the author got the impression that the spreading of the disease from plant to plant—and in certain cases also from crop to crop—must be taking place in the propagation bed and/or during the preceding treatment of the cuttings with the so-called "rooting stimulants". It was thus natural to assume that bacteria from diseased cuttings made their way out into the water in the propagation bench or into the solution of the rooting stimulant and were absorbed by adjacent, healthy cuttings with these liquids. This assumption was borne out, inter alia, by the fact that on the propagation bench, besides the diseased plants occurring sporadically, it was often possible to point out plants in distinct lots corresponding to the number of cuttings which had been treated with the same portion of "rooting stimulants"[1]).

To obtain more detailed knowledge of the dissemination of the disease, some preliminary tests were made with a view to investigating the anatomy of carnation stems and the ability of the cuttings to take up both water and bacteria. In addition, some infection tests were carried out in which the cuttings were inoculated by being allowed to take up bacterial suspensions.

b. The Anatomy of Carnation Stems

In dealing with the problem of the ability of the cuttings to take up bacteria into the vessels of the stem it was of interest to ascertain whether the vessels contained debris of transverse walls, contractions or tyloses

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[1]) It should be observed that the treatment with a "rooting stimulant" in the said nursery was carried out in the following way that 50 or 100 cuttings were placed at a time in shallow glass dishes containing a solution of a "rooting stimulant", which was used only once. After each bundle of cuttings had been treated, all the dishes were thoroughly washed, and fresh solution was poured into them for the succeeding cuttings. The "rooting stimulant" was a solution of β-indolyl-acetic acid or similar growth-promoting substances.

constituting purely mechanical obstacles to the absorption of bacteria, or whether the vessels were actually widely open tubes giving free access to the passage over long stretches for particles of the size of bacteria. In order to elucidate these questions, a number of longitudinal sections of the basal portion of the stems of healthy carnation cuttings were made (Fig. 7). All the sections showed, however, that, although the vessels of the stems are built up of cells of greatly varying lengths (most frequently from 0.5 mm to about 10–20 mm), they appear in the lowermost part of the stem as long, straight tubes without contractions or tyloses. Moreover, the vessels usually measure about 10–18 μ in diameter, so that the cross sectional area of the vessels is ample to allow even a large mass of bacteria to pass.

c. Ability of Carnation Stems to Absorb Pure Water

Further, it was of interest to find out whether the absorption of water by the cuttings is so vigorous that the bacteria can be carried along with the flow of water up into the stems. It was therefore decided to investigate how much water a cutting takes up through the base of the stem, and how far up the stems the bacteria are conveyed.

To this end, some healthy cuttings of "White Sim" and "Petersen's Pink Sim" were procured. Immediately on being broken off from the mother plants, the cuttings were placed in 1-litre beakers, 25 cuttings

Fig. 7. Longitudinal section of a carnation stem showing that the vessels may be long, unseptated tubes (about 10–18 μ diameter). ×200.

Table I. Water absorption of carnation cuttings, measured

Remarks Test period: Aug. 16–27	No. of cut-tings	Name of variety	Weight in grammes		1st day (17–18 °C)		2nd day (18–19 °C)		3rd day (19–20 °C)	
			total	per cut-ing	total	per cut-ing	total	per cut-ing	total	per cut-ing
Plants exposed	25	White Sim	182.0		39.0		32.0		43.0	
to normal day	25	–	191.5		40.0		31.5		47.0	
& night	25	–	195.5		31.5		28.0		42.0	
Average	25	–	189.7	**7.6**	36.8	**1.5**	30.5	**1.2**	44.0	**1.8**
		Petersen's								
	25	Pink Sim	149.5		28.5		25.5		42.5	
	25	–	145.0		27.5		24.0		43.3	
	25	–	145.0		26.0		23.5		40.0	
Average	25	–	146.5	**5.9**	27.3	**1.1**	24.3	**1.0**	42.0	**1.7**
Plants in dark	25	White Sim	193.0	**7.7**	25.5	**1.0**	16.0	**0.6**	15.0	**0.6**
for 7 days, then		Petersen's								
normal day & night	25	Pink Sim	153.0	**6.1**	13.5	**0.5**	9.0	**0.4**	10.0	**0.4**

in each. Into each beaker 400 ml distilled water was poured, which was replenished—gradually as the cuttings absorbed the water—with 100 ml water, so that the cuttings constantly had about 1–2 cm of their stem bases immersed. The water in each beaker was covered with an about 0.5 cm thick layer of medicinal paraffin oil to prevent evaporation from the surface.

The tests were carried out in an ordinary, light laboratory facing east, the temperature of which generally varied between 16 and 21°C, and where day and night alternated normally (August). The author, is, however, aware that the tests can only be carried out accurately in a room with constant temperature, relative humidity and known intensity of light, but such a room is not available in Denmark. The water absorption of the cuttings was therefore measured only by the daily loss in gross weight for each lot of 25 plants; the data of the tests are recorded in Table I. It may be seen from the table that under the prevailing conditions the cuttings, which weighed 5.9–7.7 g each on an average, absorb about 1.0–1.8 ml water each on an average in the first 3 or 4 days, the cuttings of the smallest weight absorbing less water than the heavier ones. After 8–9 days, the absorption of water per cutting had fallen to a little more than half a millilitre per day, presumably because the stomata were gradually closed, and perhaps also because the callus formation at the base had begun closing the stem base.

in grammes per 24 hours for 10 consecutive days.

4th day (19–21 °C)		5th day (19–20 °C)		6th day (19–20 °C)		7th day (18–19 °C)		8th day (16–18.5 °C)		9th day (16–17.5 °C)		10th day (16–17 °C)	
total	per cut-ing	total	per cut-ing	total	per cut-ing	total	per cut-ing-	total	per cut-ing	total	per cut-ing	total	per cut-ing
38.0		26.0		23.0		24.0		16.0		13.0		12.0	
46.5		34.5		28.5		30.5		20.0		17.0		16.0	
44.0		32.0		28.0		28.0		19.0		15.5		15.0	
42.8	1.7	30.8	1.2	26.5	1.1	27.5	1.1	18.3	0.7	15.2	0.6	14.3	0.6
43.5		30.0		26.5		26.5		18.0		16.5		14.5	
34.0		25.5		22.5		23.5		15.0		13.5		12.5	
36.5		25.5		21.0		22.5		14.0		12.5		12.5	
38.7	1.6	27.0	1.1	23.3	1.0	24.2	1.0	15.7	0.6	14.2	0.6	13.2	0.5
16.5	0.7	14.0	0.6	12.5	0.5	13.5	0.5	21.5	0.9	19.5	0.8	17.5	0.7
13.0	0.5	11.5	0.5	10.5	0.4	11.5	0.5	19.5	0.8	20.5	0.8	19.0	0.8

For the first 7 days of the test period some of the cutting (25 of each variety) were completely blacked out, and in these cuttings the absorption of water was immediately considerably lower than in the cuttings exposed to light during the normal length of day. The water absorption rose, however, to the same extent as in the other test plants when the black-out was lifted. This tends to indicate that the reduction in the water consumption is due, among other things, to the fact that the stomata of the cuttings close in darkness so that the evaporation through them is hampered. It was tried to determine the degree of opening of the stomata by the ordinary infiltration method, but this was not feasible with the thick, wax-clad leaves of carnations.

d. Ability of Carnations to Take up Bacteria in Aqueous Suspension

The experiments described above having shown clearly that unrooted carnation cuttings absorb rather a considerable amount of water, and that there is presumably nothing to prevent bacteria passing freely into the vessels of the carnations, some tests were finally made to see whether the bacteria are actually taken up into the vessels and, if so, how far the bacteria are conveyed up the stems. The investigations were carried out as follows: 10 cuttings of the variety "Petersen's Pink Sim" were placed in a 250 ml beaker into which a suspension of *Ps. caryophylli* (isolate

No. 83) in 50 ml water was introduced. A corresponding test was made with another 10 cuttings of the same variety and a suspension of *Serratia marcescens* Bizio. As far as practicable, the suspensions used were made with equal turbidity, and had a faintly "milky" appearance.

The reason why *Serratia marcescens* was included in the tests was the desire of working with a species of bacterium which was immediately recognizable and which might be assumed not to have any particular affinity to carnations.

The tests were carried out in a greenhouse at a temperature of 20–22°C, and the plants were left in the bacterial suspensions for 2 days. Then all the cuttings were taken out and from each cutting the lowermost 2 cm of the stem were cut off in order to make sure that a somewhat larger piece of the stem was cut off than had been immersed in the bacteria-containing water. Finally, all leaves were cut off, and the "bare" stems were then sterilized superficially by being thoroughly washed in 70% ethanol — 2‰ 8-hydroxy-quinoline sulphate $((C_9H_6(OH)N)_2H_2SO_4)$. The actual investigation as to where in the stem the bacteria were found was carried out as follows: for each 2 cm up the stem a thin slice ("disc") of about 2–3 mm was cut off under aseptical conditions and placed on PDA, after which the medium was incubated at 25–27°C. After a week, the result of the test was assessed, and it then appeared that both species of bacterium were taken into the stems almost to the same level and that all the cuttings had taken up bacteria at least 4 cm up in the stems. In several instances bacterial colonies of the said species were even found in the discs cut off from the stems at heights of both 6 and 8 cm from the original base of the cutting. All discs of stem cut off at heights exceeding 10 and 12 cm from the bases remained sterile. Later on, the experiment was repeated with a 24 hours' "treatment" and only with *Ps. caryophylli*, but it confirmed largely the first experimental results: in the repeated test the abovenamed species of bacteria were found in the carnation stems at heights of between 6 and 9 cm (Table IV. p. 57).

e. Infection Tests With *Pseudomonas caryophylli*

Since there could no longer be any doubt that carnation cuttings are capable of taking bacteria into their vessels, a smaller infection test was made, in which 3×10 cuttings of the variety "Petersen's Pink Sim" were used. The cuttings of one lot of 10 were inoculated in the same way as in the test described above, viz. by being left for 2 days in 250 ml beakers, each containing a 50 ml aqueous suspension of *Pseudomonas caryophylli* (isolate No. 83). The second lot of 10 was placed in sterile,

distilled water together with 5 infected cuttings of the same variety from which *Ps. caryophylli* had been isolated with certainty. As a check, the third lot of 10 cuttings was placed in sterile, distilled water. After 2 days, all the plants were inserted in sand, each in a sterile flower-pot of its own with autoclaved sand, and left in a greenhouse at a temperature of about 20–22°C[1]). After 6 weeks many of the cuttings inoculated with bacteria had wilted and the test was concluded. All the cuttings which were inoculated with the pure bacterial suspension of isolate No. 83 had withered completely, and the corresponding lot, which had been left in water together with diseased cuttings, did not fare any better. Of this lot, 8 plants had wilted completely in consequence of attacks by bacterial stem-crack, while 2 seemed to have entirely avoided infection. From 3 cuttings of each of the two lots of withered plants a check isolation was made, which proved the presence of *Pseudomonas caryophylli* in all 6 plants. All the check plants were, furthermore, cut up and examined under the microscope, but they were all found to be perfectly healthy. The supposition that the spreading of the disease may take place partly while the cuttings are immersed in the solution of a rooting stimulant and partly on the propagation bench thus seems to have been confirmed.

5. Description of the Isolated Bacterium

A. Morphology and Staining Reactions

The isolated bacterium is rod-shaped, though most frequently slightly curved[2]), and has rounded ends (Fig. 8). BURKHOLDER (1942) in his original diagnosis indicates that the bacterium measures $0.35–0.95 \times 1.05 \times 3.18\,\mu$, which is in excellent agreement with the dimensions ($0.5–0.7 \times 1.4 \times 3.0\,\mu$) found by the author when measuring cells in Congo red after cultivation on PDA. BURKHOLDER further states that the bacterium is provided with one or many polar or bipolar flagella, which the author is also, largely, able to confirm. However, more than 1–5 flagella at either end of the bacterium has never been found under the electron microscope (Fig. 8).

Staining of the flagella of the bacterium was attented with great difficulties. Neither the staining methods of VILH. JENSEN (1931), GRAY

[1]) Generally, this temperature is too high for carnation cuttings, but in the circumstances it was impossible to find a room that was better suited for the cultivation of carnation cuttings.

[2]) The bacterium has a striking resemblance to *Spirillum*!

Fig. 8. *Pseudomonas caryophylli* (BURKHOLDER) STARR & BURKHOLDER. Electron-photomicrograph shaded with palladium. (See also Fig. 22 on p. 110). Note the number of flagella (compare Fig. 9). Phot. A. Birch-Andersen, M.Sc., Statens Seruminstitut, Copenhagen, Denmark. ×20500.

(1926), LEIFSON (1930), or MARTIN KRISTENSEN (1955) gave satisfactory results. Some success was, however, obtained with a single preparation, by means of the so-called "silver-sulphate method" (ZETTNOW after MARTIN KRISTENSEN (1955)), but the bacteria appeared to have too few flagella (Fig. 9). When stained according to the other methods mentioned, the flagella appeared constantly as short, much—but regularly—curved filaments; their starting and terminal points, and consequently also the numbers of flagella, were quite indeterminable. In the end, it was therefore natural to see if it would be possible to use an electron miscroscope for the demonstration of the flagella of *Ps. caryophylli*. This method was not infallible either; the strange "deformities" mentioned above were also noticeable in many of the electron-micrographs; there were, however, also many excellent photographs which showed clearly the position, size and number of the flagella[1]).

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[1]) The preparations for the electron-microscopy were made in the following way: the bacteria, which had been cultivated on PDA, were first suspended in sterile, distilled water, after which the suspension was filtered through a collodion film on agar. In this way bacteria and flagella were spread uniformly over the collodion film, which was later on transferred to a brass gauze on which shadow-casting with palladium (angle 5:1) was done.

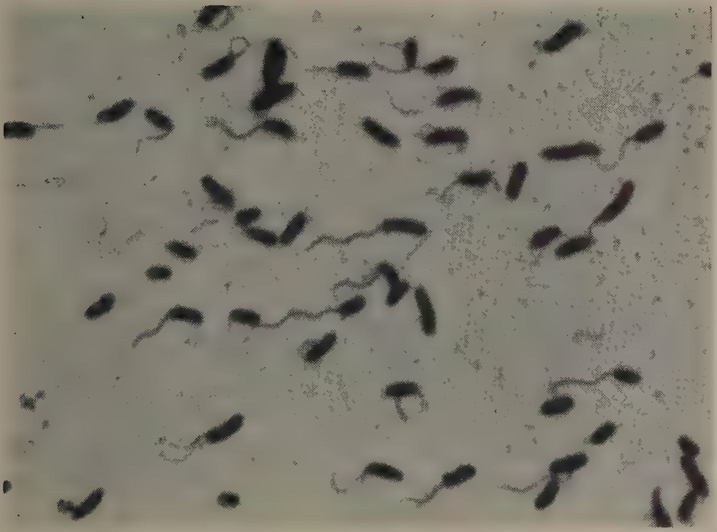

Fig. 9. *Pseudomonas caryophylli* (BURKHOLDER) STARR & BURKHOLDER, stained by MARTIN KRISTENSEN's "silver-sulphate method". Note that the bacteria when stained by this method appear with only one or a few flagella. ×2000.

The bacterium is Gram-negative and stains readily with carbol fuchsin and crystal violet.

B. Cultural Characters on Different Media

Nutrient agar (Difco) is not a very suitable medium for *Ps. caryophylli*. After 24 hours, growth is observed, but after 4 days at a temperature of 28–30°C the bacterium forms only small (1–2 mm diam.) entire-margined, pulvinate, yellow colonies. After 6 days, most of the bacteria die, and after altogether 8 days it will in most cases be impossible to make successful transfers. (cf. PDA).

Nutrient broth (Difco), unlike nutrient agar, is an excellent medium for *Ps. caryophylli*. Already after 12 to 24 hours at a temperature of 28–30°C a very distinct turbidity occurs as the result of an abundant bacterial growth. In this medium the bacterium is able to keep alive for over one month.

Potato dextrose agar (PDA) (Difco) has so far proved to be the best agar medium for *Ps. caryophylli*. After the streaking-on to PDA at 30°C of an aqueous bacterial suspension, the first colonies do not appear until after 36–48 hours. The colonies are then almost cream-coloured,

finely granular, shiny and pulvinate. After 3–5 days the centres of the colonies assume a faintly brownish hue which turns deep dark-brown in, altogether, 6–7 days. Now each individual colony measures 2–3 mm in diameter, has a narrow, light, dirty-white margin and is, moreover, so tough that most of the colony often sticks to the inoculation needle when a transfer is made. The dark-brown colour of the colony grows more intense after a couple of weeks, when it may also get a faint, mauve hue on the surface, caused by a very thin, whitish "covering layer" over the brown, central part of the colony.

The brown-colouring of the colony does not occur at all on ordinary nutrient agar (Difco), but if 1 or 2% glucose is added to the nutrient agar, the colonies on this medium will also assume a dark-brown colour, which is even darker than that of the colonies on PDA. Moreover, the addition of sugar has the effect that the bacterium grows better and that it may keep alive somewhat longer in nutrient agar.

On PDA *Ps. caryophylli* is usually able to keep alive for about one month, but if the cultures on PDA are kept in test tubes and under the cover of ordinary, sterile, medicinal paraffin oil, the bacteria will withstand storage at 15–20°C for a little more than 6 months. After 8 months, however, about half of 50 test-tube cultures had died.

Sterile potato plugs form an excellent medium for the bacterium, which forms slimy, brownish-grey growth on it. Also on this medium the bacteria will keep alive for one or two months, but after that time the potato plugs dry up to such an extent that any further storage is useless.

Nutrient gelatin (Difco) is indicated in the original diagnosis to be liquefied after 3–4 weeks, but repeated cultivating tests on Difco's nutrient gelatin have failed to confirm this. Even after altogether 2 months' storage, nothing of the gelatin was liquefied. Later investigations showed, however, that even an observation period of 3–4 weeks was useless, because also on gelatin the lifetime of the bacterium is very limited.

In autoclaved (3 hours), moist garden soil (in test tubes) at 10°C *Ps. caryophylli* keeps well for one year or more.

C. Influence of Temperature on Growth of the Bacterium

The optium temperature for *Ps. caryophylli* when cultivated on PDA in test tubes was determined to be 30–37°C. At 30, 33, 35, and 37°C the growth of the bacteria was equally abundant and could be seen

plainly on the slanting agar already after 24 hours. At 20°C and 45°C in the same period the growth was much weaker, though discernible,—particularly at 20°C, whereas at 45°C growth appeared only in about half of the tubes. At 15°C the bacterial colonies were visible (very faintly) only after 15 days, and at 5, 10, and 50°C it was impossible, even after 3 weeks' storage, to observe bacterial colonies on the medium. From cultures which had been stored for 5 days at 5, 10, and 45°C, respectively, transfers were made to test tubes with fresh PDA at 30°C, but very few colonies appeared, which seems to indicate that some of the bacteria are unable to subsist after 5 days on PDA at 5, 10, and 45°C, respectively. The thermal death point was determined by heating 1 ml of a 12 hours' culture of *Ps. caryophylli* in nutrient broth (Difco). The broth was kept in 9 mm wide but thin-glassed test tubes and the heating was done in a water bath. After 10 minutes' heating at 53°C no growth was found when transfers on to PDA and nutrient agar were made.

Thus, the temperature requirements of the Danish isolates agree very well with American descriptions, where the maximum temperature has been determined to be 46°C, the minimum temperature about 10–15°C, and the optimum temperature 30–33°C.

D. Biochemical Reactions

The ability of the bacterium to ferment carbohydrates, alcohols and glucosides was tested by cultivation in a liquid, inorganic medium[1]), to which was added 1⁰/₀ of the carbon compounds in question; the medium was autoclaved at 110°C for 10 minutes. Bromthymol blue was used as indicator, and the hydrogen-ion concentration of the medium was adjusted to pH 7.2. After 3 days in incubator at 28–30°C, acid had been formed (the colour of the medium changed from greenish-blue to yellow), but no gas was formed in the tubes with glucose, fructose, xylose, galactose, mannose, saccharose, sorbitol, and arabinose. After 8 days, also raffinose, mannitol, and glycerol could be included among the carbon compounds which can be utilized by *Ps. caryophylli*. Lactose and maltose are fermented very slowly, the change in colour of the medium being ascertained only after 3–4 weeks. Salicin, dextrin, and inulin were not fermented even after 2 months. In BURKHOLDER's description salicin was stated to be fermented, but this must be due to a misprint or perhaps to

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[1]) Formula (after AYRES et al. 1919): $(NH_4)H_2PO_4$ 1.0 g; KCl 0.2 g; $MgSO_4$ 0.2 g; H_2O, dist. 1000 ml.

contamination during the testing, since so far none of the plant pathogenic *Pseudomonas* species have been able to ferment salicin, and in the author's tests neither an American nor the Danish isolates of *Ps. caryophylli* were able to ferment this glucoside.

The diastatic reaction of the bacterium was examined by cultivation on nutrient agar + 0.2% water-soluble starch, but after 2, 3, and 4 weeks, none of the starch had been hydrolyzed by testing with Lugol's liquid.

In Difco's nutrient broth a small amount of ammonia was produced after 8–10 days (Nessler's reagent), and in Difco's nitrate broth a slight formation of nitrite could be ascertained by testing with Gries's reagent after 10 days at 28–30°C.

The test for indole formation was made by cultivation in a trypsin-casein solution and a 1% Difco bacto-tryptone solution. After 2, 5, 10, 20, and 30 days, additions of paradimethylaminobenzaldehyde dissolved in amyl alcohol and concentrated hydrochloric acid were made, but no red-staining of the reagent was discernible.

The hydrogen sulphide reaction was also negative. For this test the bacterium was cultivated on Difco's "iron-agar", but after altogether 4 weeks no brown-staining of the medium was traceable. Lead acetate paper suspended over cultures grown on PDA, nutrient broth and sterile potato plugs likewise remained unstained.

For the purpose of Voges-Proskauer reaction and the methyl-red test, the bacterium was cultivated on Difco's M.R.-V.P. medium, but both tests were negative after 3 days.

Neutral litmus milk (Difco) became slightly alkaline after 1–2 weeks at 30°C, the medium assuming a dirty, bluish-grey colour. After 3 weeks, a whitish precipitate had been formed, which just filled out the round bottom of the tube, but the milk was not turned into soft curd. After 2 months the liquid was still dirty-bluish, but a lilac-coloured clearing of the uppermost 5 mm of the liquid had begun. This clearing (peptonizing) continued, as far as the American isolate was concerned, until the whole of the medium (about 5 ml) had become peptonized in the course of 4 months. In the same period, the Danish isolates had only produced a clearing of the uppermost 10 mm of the litmus milk.

All the cultivation tests included two Danish isolates (Nos. 83 & 84) and an American isolate, which the author had received through Dr. W. J. Dowson, Cambridge, England, as a check isolate. The tests with each isolate comprised 4 test tubes of each medium. All the tests were performed 3 times in all. (Cf. p. 14, upper part).

6. Dissemination of the Disease

Bacterial stem-crack of carnation is a pronounced vascular disease and so it is mostly confined to the vessels of the plant or to the cavities arising in the stems when, in the case of heavy attacks, the bacteria have destroyed completely the vascular tissue. Only where the disease causes the stems to burst may bacterial slime be exposed to the atmosphere, thus creating a possibility of direct transfer of infection to other plants. The bacterial slime is, however, very sticky, and as the parts of the plant which are above ground have to be kept as dry as possible to prevent attacks by fungi (*Fusarium*, *Uromyces*, etc.)—and are, in fact, generally kept very dry—the risk of transfer of the sticky bacterial slime to other plants is but slight. Furthermore, the stems are undoubtedly able to be infected only through wounds, a fact which was observed during the infection tests described above, in which the wounded carnation stems were inoculated by being smeared with bacterial slime both into the pricked wounds and on a large area around them. It was, however, never possible in these infection tests to demonstrate any symptom of bacterial attack around the wounds, and so the chance of direct communication of the disease from one diseased stem to other, unwounded, stems seems to be rather slight—at any rate under Danish conditions. This assumption also agrees very well with practical experience, which shows that the young cuttings are infected during the root-forming process and that the diseased plants in a certain crop immediately, or at any rate in the course of the first year, appear to be scattered rather uniformly all over the beds; then the spreading of the disease takes place very slowly and apparently only from one root system to another.

Infection tests (cf. Sect. 4 A b, p. 28)—and also some practical experience —have shown that infection through the roots very easily takes place, but although a great many bacteria are supplied to the soil from the infected and decaying roots, the disease seems to advance remarkably slow in the Danish carnation beds. This may presumably be explained by the fact that the soil temperature in Danish carnation beds generally is too low for normal growth of the bacterium. The soil temperature in our carnation beds will but rarely exceed 10–15°C, and as *Ps. caryophylli* grows only with difficulty at temperatures below 20°C, the bacterium must have poor living conditions in the Danish carnation beds; but more intricate microbiological processes may, of course, also be assumed to hamper the growth of the bacterium.

Bacterial stem-crack thus is no epidemic disease in older carnation

crops in Denmark. During the propagation by cuttings, the disease may, however, assume an epidemic character, and there can hardly be any doubt that the spreading of bacterial stem-crack from plant to plant takes place primarily at this early stage of the life of the carnation.

Thus, the actual source of infection of bacterial stem-crack is the infected cuttings which are taken from diseased, but healthy-looking, mother plants. This is possible because the bacteriosis often attacks only one or a few vessels in the carnation stems (Fig. 1, p. 17), and because such slightly infected plants generally do not present outward symptoms of the disease. Yet the disease is present in the vascular system of these plants, and the cuttings which have direct contact with the infected vessels of the mother plant will usually also carry the infectious matter in their vessels. If attacks of bacterial stem-crack are present in a certain carnation crop, it is generally not possible to avoid that cuttings with infected vessels are selected and inserted in sand together with entirely healthy ones. This, again, will have the effect that bacteria from the diseased vessels make their way to the water in the propagation bed, from where they are taken up by adjacent healthy cuttings. The cuttings may, however, also be infected already before being placed in the sand of the propagation bed, for instance during the treatment with "rooting stimulants" or by being left standing in water, which is a practice rather commonly adopted to prevent too rapid drying-out of young shoots. Whether the plants are stood in the solution of a rooting stimulant or in ordinary tap-water, bacteria will in any case seep out from the vessels of the diseased cuttings and be absorbed by healthy cuttings, which are thereby inoculated more or less thoroughly.

The course of the disease is then dependent partly on the number of vessels infected and partly on the temperature. A high temperature (i. e. 20–25°C and above) furthers the growth of the bacteria, while carnations in this country develop best at relatively low temperatures. Accordingly, a high temperature during the propagation and in the first time after planting-out will tend to accelerate the disease and in several cases kill cuttings or plants quickly. At lower temperature, i. e. about 10–16°C, many slightly infected cuttings will often be able to keep alive with a normal appearance for several weeks or months, and it is from these plants that there is a particularly great risk of taking infected cuttings.

7. Control

A. Direct Control Measures

a. Measures to Prevent Spreading of the Bacteria During the Propagation by Cuttings

Since bacterial stem-crack of carnation in Denmark is almost exclusively disseminated during propagation by cuttings, it is at once suggested to control the disease primarily by preventing the bacteria from being transferred from one cutting to another. The first time such a transfer of bacteria from diseased cuttings to healthy ones can take place is immediately after the breaking-off of the cuttings, when they are to be treated with a so-called "rooting stimulant". It should be observed that it is the general practice in this country to treat the cuttings with such a preparation before they are inserted in the sand bed. The preparation usually contains β-indolyl-acetic acid or the like, which, when used in the proper concentration, accelerates the root formation of the cuttings. Such preparations are available either as a powder to be applied by powdering or as a solution. Though these forms of the preparation have largely the same effect, growers often prefer one or the other of them. If, however, the application of a solution of a rooting stimulant is not insisted upon, it is possible, by using the dry powder, to avoid dissemination during this treatment of the cuttings. In practice, the powdering may be done simply by collecting the cuttings in bundles of 25–50 and placing them on the edge of a table so that all the cut-surfaces form an even surface protruding somewhat from the edge of the table. The rest of the bundle is then covered with a piece of paper or the like, after which the powder is applied to the cut-surfaces of the cuttings by means of a small hand-operated powder blower. In this way all the cuttings receive a very uniform quantity of the root promoting substance, and, in addition, the transfer of bacteria from diseased cuttings to healthy ones is entirely avoided. Each cutting may, however, also be dipped directly into the powder (in a shallow dish), and also this method ensures that the bacteria are not transferred from one plant to another, but it is a more time-consuming method, and the powder is not applied so uniformly as when a powder blower is used.

If, however, a solution is used, the bacteria will make their way from the attacked vessels into the liquid, in which they will be distributed evenly in the course of the treatment period—usually from 12 to 24 hours—and be taken up by the healthy cuttings together with the liquid,

and these cuttings will thus be particularly thoroughly inoculated. It was especially in this way that the bacterium spread in the Copenhagen nursery where the disease was first observed. As, however, the nursery was very reluctant to replace the liquid treatment with the powdering, it was tried whether, by adding a disinfectant to the solution of the rooting stimulant, it was possible to prevent dissemination through this channel.

With this end in view, some preliminary tests were made, in which *Ps. caryophylli* was cultivated in nutrient broth with a little glucose added, as well as various quantities of the bactericide 8-hydroxy-quinoline sulphate ($(C_9H_6(OH)N)_2H_2SO_4$). The tests were carried out in the following way: first, 3 litres nutrient broth (Difco) was made, to which $1^0/_0$ glucose was added. This quantity of liquid was then distributed into 10 Erlenmeyer flasks, to which further 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and $1.0^0/_{00}$ 8-hydroxy-quinoline sulphate, respectively, were added. The liquids were then poured into test tubes, 10 ml in each, and altogether 10 test tubes of each concentration were used. Besides, the test included 10 "check tubes" with nutrient broth + $1^0/_0$ glucose without addition of any disinfectant. Finally, all the tubes were inoculated with 5 drops of an aqueous suspension of *Ps. caryophylli* (isolates Nos. 83 & 84 mixed). The tubes were not plugged but exposed to the atmosphere in an ordinary laboratory at a temperature of 18–22°C. Efter 24 hours, all the check tubes, as well as the tubes with $0.1^0/_{00}$ 8-hydroxy-quinoline sulphate, were heavily turbid with bacterial growth, while but few tubes with $0.2^0/_{00}$ 8-hydroxy-quinoline sulphate displayed a slight bacterial growth. All the remaining tubes, with the concentrations 0.3 to $1.0^0/_{00}$ 8-hydroxy-quinoline sulphate, remained sterile throughout the two months of observation.

It was therefore reasonable to assume that addition of 8-hydroxy-quinoline sulphate to the solution of rooting stimulant was able to kill all the bacteria oozing out from diseased cuttings, but the question then remained whether the carnation cuttings would stand such treatment. To have this question elucidated, an experiment was made with 8-hydroxy-quinoline sulphate treatment of altogether 3000 cuttings of a lot of the variety "Harvest Moon", about which it was known that only a small percentage was attacked by bacteriosis. The test plants were divided into 6 lots, each of 500 cuttings, of which one lot was left untreated, while another was treated with the usual rooting stimulant. The remaining 4 lots were treated with a solution of rooting stimulant to which was added 0.3, 0.5, 0.75, and $1.0^0/_{00}$ 8-hydroxy-quinoline sulphate,

respectively. All the plants were left for 16 hours in the respective liquids, after which they were inserted in sand in the usual manner on an ordinary propagation bench. As a check on the sterility of the liquids used, a 5 ml sample of each category was taken out after the treatment and immediately streaked on to PDA in Petri dishes. These tests showed, in the course of a week's time, that all the liquids which contained 8-hydroxy-quinoline sulphate were completely sterile, whereas in the samples of the check liquids—partly ordinary water, partly water + rooting stimulant—numerous colonies of bacteria were found—also a few of *Ps. caryophylli*. After about 3 weeks all the cuttings were taken out, and it then proved that all the cuttings were able to stand even the strongest treatment ($1.0^{0}/_{00}$) without showing the least sign of injury. The test was immediately repeated with the same liquid concentrations and the same number of cuttings of each of the varieties "Petersen's Pink Sim", "White Sim", and "Salmon Sim". But in these tests certain of the categories of cuttings—as far as could be judged, the most herbaceous ones—were "burned" (black stained) on the cut surfaces, and, besides, had lost some of their turgescence. Furthermore, their root development was somewhat retarded, especially in the case of an 8-hydroxy-quinoline sulphate addition of between 0.5 and $1.0^{0}/_{00}$. It was therefore decided only to use $0.3–0.4^{0}/_{00}$ 8-hydroxy-quinoline sulphate in the root-promoting solution in future, and about 30,000 cuttings were treated with this mixture in 1954 without any essential drawbacks, and random samples of the liquids were always found to be sterile. However, also with these low concentrations, a faint black-staining of the cut-surfaces might occur now and then, but the root formation was not affected in any degree worth mentioning. Hence, it can be said with certainty that if the application of a solution of a rooting stimulant instead of powder is insisted upon, such a solution may be used without any danger of transfer of disease, provided 0.3 to $0.4^{0}/_{00}$ 8-hydroxy-quinoline sulphate is added.

Concurrently with the above mentioned tests, exactly the same test series were made with the addition of "Aretan" ($4.5^{0}/_{0}$ alkoxy-ethyl-mercury compound) in lieu of 8-hydroxy-quinoline sulphate, but in order to obtain the same bactericidal effect in the liquids it was necessary to use "Aretan" in double strength, i. e. at least $0.5–0.6^{0}/_{00}$, and when treated with "Aretan" in these concentrations all the cuttings were killed.

The next stage in the propagation of cuttings during which the bacteria may be transferred from diseased cuttings to healthy ones is the sand bed, where the carnations usually spend 2 to 3 weeks until the newly

formed root system has become so well developed that the young plant may be moved (despatched and planted out) without suffering any harm. Prevention of spreading of the bacteria in the sand is, however, hardly possible at the time being. In the first place, the chemicals which might be used at present, e. g. mercury compounds and 8-hydroxy-quinoline sulphate, would damage the cuttings during the long treatment period required. Secondly, it would be too costly continually to sprinkle or spread disinfecting chemicals. Finally, it should be borne in mind that, judging from all appearances, such a control is of little use if the infected cuttings cannot be cured simultaneously. This control has, however, so far proved to be unfeasible, and, despite the application of strong disinfectants, even the slightly infected carnation cuttings will therefore continue their more or less languishing growth and, eventually, carry the disease into the greenhouse, where the plants will either die and infect other carnations, or, what is still more detrimental, persist with infected vascular tissues, through which the bacteria may reach the next generation of cuttings and thus contribute towards spreading the disease.

Any direct control of the bacteriosis while the cuttings are in the propagation bench must therefore—at the time being—be said to be out of the question. At this stage, efforts should be made to find a method by which the diseased cuttings can be found and destroyed before the actual setting takes place (see Sect. 7B: Indirect control measures, p. 70).

If, nevertheless, the bacteriosis occurs among the cuttings during the root-forming period, both the sand and the boards of which the bench is usually built up will become saturated with bacteria-containing water, and experience shows that both sand and boards are capable of conveying the disease to new crops (see Sect. 4Ab, p. 28). There can therefore be no doubt that in order to avoid infection from one lot of cuttings to another, it will be necessary to renew the sand (or whatever medium is used) completely or, at any rate, subject the medium in question to a very thorough steaming whenever a new culture of cuttings is commenced. The bench materials will likewise have to be replaced or steamed after each crop, because even a very thorough soap-washing with subsequent immersion in an "Aretan" solution and painting of the boards has proved to be insufficient disinfection! Boards are very difficult to sterilize and should for this reason either be avoided completely or replaced with entirely new ones for each crop of cuttings. This method will usually be a very expensive one, and it is therefore advisable to build up the propagation benches of less porous materials, e. g. asbestos-

cement sheets "Eternit" or "Everite", which are easily sterilized by means of steam or by immersion in disinfectants, for which reason they can be used for a long time without any risk of communicating infection.

b. Control by Means of "Aretan" and 8-Hydroxy-Quinoline Sulphate

Since, in the earliest stages of the disease, the infectious matter of bacterial stem-crack is strictly confined to the vessels of the plants—often to only a few vessels (Fig. 1, p. 17)—, it would be tempting to try whether the disease could be controlled in cuttings newly broken off, by letting the cuttings absorb a bactericide so that the bacteria might be killed in this way in the infected vessels. Actual curing of vessels already attacked was, of course, out of the question, but the disease might perhaps be checked completely in this way, and so this method would also constitute an acceptable disease control. The question was, however, whether bactericides could be found which were non-toxic to carnations and also could be absorbed by the stems of the plants. As far as the author was able to judge, three possibilities presented themselves in the first place, viz. the organic mercury compounds, 8-hydroxy-quinoline sulphate, and antibiotics.

The chance of using agents containing mercury seemed, however, a priori to be slim, since experiments already carried out with treatment of carnation cuttings with aqueous solutions of "Aretan" (see p. 45) had proved that, even in weak concentrations, this preparation was extremely toxic to carnations. On the other hand, treatment of numerous cuttings with aqueous solutions of 8-hydroxy-quinoline sulphate had proved to be relatively harmless to carnations (see p. 45), and as, moreover, this substance had proved to be very toxic to *Ps. caryophylli* (p. 44), there would thus appear to be rather good possibilities of using 8-hydroxy-quinoline sulphate as a systemic-acting means of control. For the applicability of antibiotics as systemic-acting bactericides, reference is made to Sub-sect. c, p. 49.

Before detailed investigations of the ability of carnation cuttings to absorb the various chemicals were commenced, another testing of the toxicity of "Aretan" to carnation cuttings of the varieties "Petersen's Pink Sim", "White Sim", and "Harvest Moon" was made. For this test, three different aqueous "Aretan" solutions with strengths of 0.25, 0.50, and 1.00$^0/_{00}$, respectively, were used. For each concentration, 100 cuttings of each of the tree varieties were selected, i. e. altogether 300 ×

3 = 900 cuttings, as well as 100 untreated cuttings of each variety. The cuttings were treated in the usual way, being placed in 1-litre beakers with 200 ml solution—i. e. a liquid level of about 2 cm in each beaker—, and after 24 hours, all cuttings were inserted in sand in the usual manner together with the other cuttings of the nursery in question. The test bore out completely the observations made previously. In about a fortnight all the treated cuttings assumed a greyish colour and withered without the slightest sign of root formation. Further experiments with this mercury preparation were therefore given up.

Next, some tests were made with a view to investigating whether different solutions of 8-hydroxy-quinoline sulphate could be absorbed by carnation stems and, if so, whether this substance was strictly confined to the vessels or was able to diffuse from the vessels into adjacent cells in the stem tissue. Particularly, the author entertained the idea that the 8-hydroxy-quinoline sulphate might be absorbed by healthy vessels and thence diffuse into the diseased, bacteria-filled vessels, killing the bacteria there.

The absorption tests were carried out in the usual way, by letting about a score of cuttings of the variety "Harvest Moon" stand in 1-litre beakers containing a 2 cm deep (= 200 ml) 0.5 ‰ aqueous solution of 8-hydroxy-quinoline sulphate, which concentration had proved, in general, to be the strongest that could be applied to carnation cuttings. After 24 hours, 10 cuttings were selected for a further investigation, which was carried out as follows: first, the lowermost 3 cm of the stem were cut off from each cutting, thus making sure that the whole of the part of the stem that had been dipped in the liquid was discarded. From the remaining part of the stem altogether 4 small, about 3–4 mm thick, discs of stem were cut off for each 3 centimetres up the stem. The first disc was cut off immediately above the newly discarded (basal) piece of stem, i. e. about 3 cm up the stem; disc No. 2 was cut off about 6 cm from the original base of the stem, and so on. These four discs were immediately placed in numerical order on PDA in Petri dishes which had just been inoculated with an aqueous suspension of *Ps. caryophylli*. The idea was to examine whether any bactericidal effect originating from the discs of stem and manifesting itself by a larger or smaller clear zone within which no bacterial growth could be demonstrated, would be noticeable.

Apart from the lowermost discs (No. 1), which had been cut off at a distance of about 3 cm from the original base of the stem and around which a very narrow clear zone occurred now and then, no bactericidal effect was found in the discs. The rest of the test plants (10) were, however,

allowed to stand for about 48 hours in the 8-hydroxy-quinoline sulphate solution, after which the same biological testing was done, but in connection with the discs of stem from these cuttings, clear zones always occurred around the lowermost disc, and sometimes also around the second one, but in the case of the 3rd and 4th discs, the bactericidal effect always failed to appear.

Apparently, 8-hydroxy-quinoline sulphate could only in a slight degree be absorbed by carnation stems, and, as a check, new absorption tests were made with some slightly infected carnation stems of the variety "Harvest Moon". The test was carried out in the same way as the one just described, with 48 hours' treatment, and it confirmed perfectly the results of the previous test, seeing that colonies of *Ps. caryophylli* appeared around most of the discs of stem—also the lowermost ones—, which were this time placed on pure PDA (not inoculated with bacteria).

Thus, the 8-hydroxy-quinoline sulphate solution had been unable to kill the bacteria in the diseased vessels, but whether this was due to the fact that neither the diseased nor the healthy vessels had absorbed the substance, or it was taken up only in healthy vessels, from which it could not get in touch with the bacteria in the diseased vessels, could not be established in this experiment. It was therefore decided to find out whether any bactericidal effect could be demonstrated in tiny prismatic pieces of pith cut from pieces of stem originating from cuttings which had been left for 48 hours in $0.5^0/_{00}$ 8-hydroxy-quinoline sulphate, the bactericidal effect of which had first been ascertained by placing the discs of stem on PDA inoculated with bacteria. The 10 tests made were, however, all negative. The author is therefore of the opinion that 8-hydroxy-quinoline sulphate is not, or only in a very slight degree, able to diffuse from healthy vessels to adjacent cells.

Nor was it possible to prove irrefutably that the diseased vessels were unable to absorb 8-hydroxy-quinoline sulphate, but experiments with the absorption of dyes, e. g. eosin, seemed to indicate that the dye was not absorbed by the vessels filled with bacteria—and it is not probable either that vessels which are more or less choked with bacterial slime would be able to take up liquids at all.

c. Control by Means of Antibiotics

i. Testing of the inhibiting effect of various antibiotics on *Pseudomonas caryophylli*. After the tests described above had shown that the control of bacterial stem-crack of carnation by allowing the cuttings

to absorb some mercury-containing agent or a solution of 8-hydroxy-quinoline sulphate was not possible for the present, it was investigated whether antibiotics had any bactericidal effect on *Pseudomonas caryophylli* and, if so, whether antibiotics could be absorbed by carnation stems.

The preliminary tests aimed primarily at testing the effect of various antibiotics on *Ps. caryophylli*, and for this purpose some common medicinal preparations containing penicillin (50 int. units), aureomycin (5 mg), polymyxin, streptomycin (5 mg), and terramycin (5 mg) were used, besides Agri-mycin 100—a mixture of streptomycin (15%) and terramycin (1.5%)—but less pure than the other substances mentioned, Agri-mycin 100 being intended solely for use in the control of plant diseases. With the exception of Agri-mycin 100 and polymyxin, the initial, preliminary, investigation was made with the application of the so-called "resistance tablets"[1]), which are used mostly in connection with medical examinations and contain, besides some filler, a certain quantity (the figures in parentheses) of the antibiotic in question. The tablets used were about 1 mm thick and 9 mm in diameter and were in the test placed on a 6–7 mm thick agar medium (PDA)[2]) in Petri dishes which had just been sown uniformly with a suspension of *Ps. caryophylli* (a mixture of isolates Nos. 83 & 84). For the Agri-mycin and the polymyxin, no resistance tablets were available, and in these cases the so-called "disc-method" was therefore used, in which small discs of filter paper (6 mm in diam.) soaked in an aqueous solution of Agri-mycin or polymyxin—in this case a 1.0‰ solution—are placed on the medium. For each of the 6 antibiotics, altogether 10 tablets (discs) were used, distributed on 2 Petri dishes (5 in each), which were incubated at 28°C. The test comprised altogether 12 dishes with antibiotics and 2 check dishes. After 2 days, the dishes were examined to see whether, around the tablets, a clear zone had appeared in which no bacterial growth had taken place, and the diameters of such clear zones were measured, the width of the zone being an expression of the antibiotic effect of the substance in question. The examination showed, however, that neither around the penicillin and aureomycin tablets nor around the polymyxin discs had any clear zones appeared, and so these substances must be considered to have no effect on *Ps. caryophylli*. However, around the streptomycin and terramycin tablets, as well as

[1]) Manufactured by Roskilde Medical Company Ltd., Roskilde, Denmark.

[2]) Nutrient agar should not be used in tests with antibiotics because its content of peptone hampers the effect of these substances (direction from Roskilde Medical Company).

around the paper discs with Agri-mycin 100, distinct, clear zones of about 4–6 mm width, and sharply outlined against the heavily developed bacterial film in the dishes, had formed. Consequently, streptomycin, terramycin and Agri-mycin 100 has a considerable antibiotic effect on *Ps. caryophylli*, and the continued investigations were therefore carried out primarily with these three substances[1]). The more detailed tests were all made with the aforementioned "disc-method", and of streptomycin (dihydrostreptomycin sulphate)[2]) and terramycin (oxytetracycline)[3]) the pure substances, as employed for medical purposes in this country, were used. These substances were furthermore tested in two different concentrations, corresponding to 0.01 $^0/_{00}$ (10 ppm) and 0.1 $^0/_{00}$ (100 ppm). As Agri-mycin 100 is a rather impure commodity intended solely for the control of plant diseases, it was preferred in this case to use the concentrations recommended in the directions for use received from America, viz. 0.7, 1.4, 2.8, and 5.6 $^0/_{00}$ of which 0.7 $^0/_{00}$ corresponds to 100 ppm in respect of content of streptomycin. (These rather strange concentrations were arrived at by converting American pounds and gallons into grammes and litres).

Besides the actual check, in which discs of filter-paper dipped in sterile, distilled water were used, polymyxin was once more included in the first large-scale tests, because it was stated to have a particularly good antibiotic effect on *Pseudomonas* species. For each concentration of each antibiotic, 10 Petri dishes with 3 discs (a, b, and c) in each were used, i. e. altogether 30 discs per concentration per antibiotic. After all the Petri dishes had been sown with a bacterial suspension of *Ps. caryophylli* (isolate No. 83), and the various discs of filter-paper had been placed on the agar, the dishes were incubated at 28°C, and after 24 hours the clear zones which in the majority of cases had formed around the paper discs were measured (the results are given in Table II).

From Table II it appears that the antibiotic effect of polymyxin on *Ps. caryophylli* is very slight and uncertain, so this substance was tested no further. Terramycin, however, had an extraordinarily powerful effect when used in a concentration of 0.1 $^0/_{00}$ (= 100 ppm), while the effect of 0.01 $^0/_{00}$ (= 10 ppm) was slight and uncertain (Fig. 10).

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[1]) Random examinations were made to see whether the bacteria in the "clear zone" were killed, or whether the various antibiotics had only a bacteriostatic effect. As the concentrations used—particularly the weaker ones,—were not always found to be bactericidal, the term "antibiotic effect" will primarily be used in the following.

[2]) Manufactured by Rhône-Poulenc, France.

[3]) Manufactured by Chas. Pfizer & Co., USA.

Table II. The inhibiting effect of various antibiotics on *Pseudomonas caryophylli*, measured at 28°C.

Name	Concentration ‰	Disc No.	Diameter of clear zone, measured in mm[1]) Dish No. 1	2	3	4	5	6	7	8	9	10	Average
Streptomycin	0.01	a	15	13	16	15	23	15	21	17	20	15	
		b	14	13	21	16	20	17	20	19	24	17	
		c	14	12	16	21	18	23	20	20	23	17	
		Average	14.3	12.7	17.7	17.3	20.3	18.3	20.3	18.7	22.3	16.3	**17.8**
	0.1	a	27	30	29	27	27	28	24	27	25	28	
		b	27	30	29	28	28	27	24	26	26	26	
		c	28	29	26	28	29	27	24	26	27	27	
		Average	27.3	29.7	28.0	27.7	28.0	27.3	24.0	26.3	26.0	27.0	**27.1**
Terramycin	0.01	a	6	6	6	6	10	6	6	6	6	6	
		b	6	6	6	6	9	8	6	6	6	6	
		c	6	6	6	6	9	9	8	6	6	9	
		Average	6.0	6.0	6.0	6.0	9.3	7.7	6.7	6.0	6.0	7.0	**6.7**
	0.1	a	30	30	28	29	25	30	30	29	30	28	
		b	28	27	31	29	23	27	29	28	29	23	
		c	28	28	29	28	22	28	28	26	30	25	
		Average	28.7	28.3	29.3	28.7	23.3	28.3	29.0	27.7	29.7	25.3	**27.8**
Polymyxin	0.01	a	6	6	6	6	6	6	6	6	6	6	
		b	6	6	6	6	6	6	6	6	6	6	
		c	6	6	6	6	6	6	6	6	6	6	
		Average	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	**6.0**
	0.1	a	9	10	6	6	9	6	6	6	6	6	
		b	6	9	10	6	6	6	6	6	6	6	
		c	6	9	9	6	10	6	6	6	6	6	
		Average	7.0	9.3	8.3	6.0	8.3	6.0	6.0	6.0	6.0	6.0	**6.9**
Agrimycin 100	0.7	a	25	26	26	25	25	25	23	23	23	26	
		b	26	26	26	25	25	24	24	25	23	26	
		c	25	28	26	25	25	25	23	25	24	26	
		Average	25.3	26.7	26.0	25.0	25.0	24.7	23.3	24.3	23.3	26.0	**25.0**
	1.4	a	26	26	26	29	24	24	25	27	26	25	
		b	26	26	26	26	25	24	27	26	27	27	
		c	27	26	26	25	25	25	27	26	28	27	
		Average	26.3	26.0	26.0	26.7	24.7	24.3	26.3	26.3	27.0	26.3	**26.0**
	2.8	a	27	28	29	26	27	29	28	28	20	25	
		b	26	28	29	27	27	29	28	30	15	27	
		c	27	31	29	28	29	28	30	27	20	27	
		Average	26.7	29.0	29.0	27.0	27.7	28.7	28.7	28.3	18.3	26.3	**27.0**
	5.6	a	30	28	30	32	29	29	30	32	28	31	
		b	27	27	29	28	28	29	29	30	29	31	
		c	29	29	32	29	30	29	31	29	30	32	
		Average	28.7	28.3	30.3	29.7	29.0	29.0	30.0	30.3	29.0	31.3	**29.6**
Check (dist. water)		a	6	6	6	6	6	6	6	6	6	6	
		b	6	6	6	6	6	6	6	6	6	6	
		c	6	6	6	6	6	6	6	6	6	6	
		Average	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	**6.0**

[1]) See note, Tab. III, p. 55.

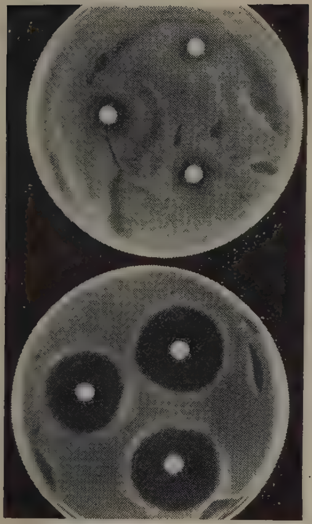

Fig. 10

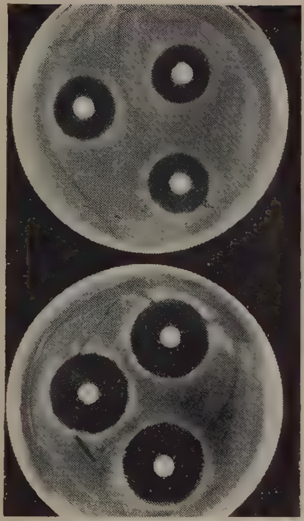

Fig. 11

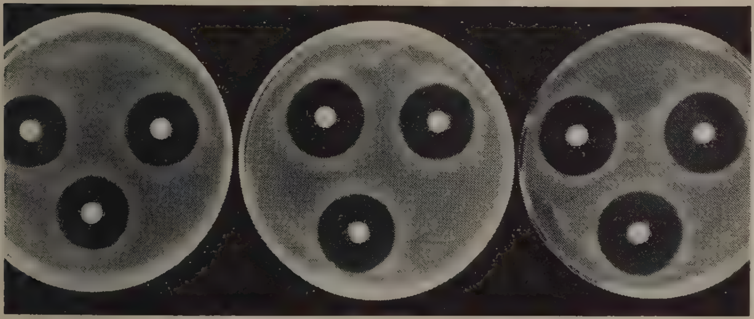

Fig. 12

Figs. 10–12. The antibiotic effect of different antibiotics on *Ps. caryophylli*. The Petri dishes are sown with a pure culture of *Ps. caryophylli*, isolate No. 83, after which 3 discs of filter-paper soaked with a solution of an antibiotic are placed on the freshly sown agar plate (PDA). All photographed after 2 days. ×0.4.

Fig. 10. Antibiotic effect of terramycin. Above: $0.01\,^0/_{00}$ (= 10 ppm), below: $0.1\,^0/_{00}$ (= 100 ppm).

Fig. 11. Antibiotic effect of streptomycin. Above: $0.01\,^0/_{00}$ (= 10 ppm), below: $0.1\,^0/_{00}$ (= 100 ppm).

Fig. 12. Antibiotic effect of Agri-mycin 100: From the left: 0.7, 1.4, and $2.8\,^0/_{00}$ Agri-mycin 100.

Streptomycin had an excellent and certain effect in both concentrations—greatest, however, in the concentration of $0.1^{0}/_{00}$ (100 ppm) (Fig. 11). A similar, definite and good antibiotic effect was found in all concentrations of Agri-mycin 100, even the weakest concentration of this substance ($0.7^{0}/_{00}$) having a considerable effect (Fig. 12).

The test represented by Table II was at once repeated in the way that a set of dishes were incubated at 22°C, and another set at 30°C. The repeated test comprised only 3 dishes per concentration at each temperature (i. e. $3 \times 3 = 9$ discs per concentration per temperature).

The results of the repeated test are listed in Table III, and hence it appears that, in general, there is good agreement with the results of the preceding test, and that the effect of the antibiotics tested generally is lower at 30° than at 28°C; on the other hand, it is higher at 22°C than at 28°C. This may presumably be explained by the fact that the substances are destroyed more quickly at the higher temperature, but the rates of diffusion and growth may, of course, also have some contributary effect.

ii. Ability of carnation cuttings to absorb streptomycin, terramycin, Agri-mycin 100, 8-hydroxy-quinoline sulphate, and *Pseudomonas caryophylli*. The results of the tests described in the foregoing section left no doubt that both terramycin and streptomycin, as well as a mixture of these two substances, as in Agri-mycin 100, had an excellent antibiotic effect on *Pseudomonas caryophylli*. The next question was whether these substances could be absorbed by carnation cuttings, and a preliminary test was then made, in which 60 healthy cuttings of the variety "Petersen's Pink Sim", were placed in 1-litre beakers, 10 in each, after which 200 ml of the liquid in question was poured into each beaker. However, as it had been established in the preceding tests that the strong concentrations had the most reliable and greatest antibiotic effect, streptomycin and terramycin were in this preliminary test used only in concentrations of $0.1^{0}/_{00}$ (= 100 ppm), while Agri-mycin 100 was used in a concentration of $5.6^{0}/_{00}$ (cf. p. 51). The test included—as a check—partly an $0.5^{0}/_{00}$ aqueous solution of 8-hydroxy-quinoline sulphate and partly sterile, distilled water. In addition, an aqueous suspension of *Ps. caryophylli* was included in order to test the absorption power of the cuttings once more. Thus, altogether 6 different liquids with 10 cuttings in each, in all 60 cuttings, were included. The test plants were left in an ordinary light laboratory at a temperature of 18–22°C, and after 24 hours, 4 cuttings were selected from each lot (beaker), after which, like in the absorption tests previously

Table III. The inhibiting effect of various antibiotics on *Pseudomonas caryophylli* at 22°C and 30°C, respectively.

Name	Concentration ‰	Disc No.	Diameter of clear zone in mm[1]) 22°C Dish No. 1	22°C 2	22°C 3	22°C Average	30°C Dish No. 1	30°C 2	30°C 3	30°C Average
Streptomycin	0.01	a	23	28	21		14	16	9	
		b	22	26	20		16	15	8	
		c	21	27	22		9	16	6	
		Average	22.0	27.0	21.0	**23.3**	13.0	15.7	7.7	**12.1**
	0.1	a	37	32	31		21	20	20	
		b	35	34	33		21	20	21	
		c	35	34	32		22	20	20	
		Average	35.6	33.3	32.0	**33.6**	21.3	20.0	20.3	**20.5**
Terramycin	0.01	a	6	6	6		6	6	6	
		b	6	6	6		6	6	6	
		c	6	6	6		6	6	6	
		Average	6.0	6.0	6.0	**6.0**	6.0	6.0	6.0	**6.0**
	0.1	a	15	16	12		25	29	21	
		b	16	13	14		26	24	23	
		c	15	15	13		25	20	28	
		Average	15.3	14.7	13.0	**14.3**	25.3	24.3	24.0	**24.5**
Agrimycin 100	0.7	a	33	26	32		21	22	20	
		b	33	26	31		20	20	20	
		c	27	26	30		19	19	20	
		Average	31.0	26.0	31.0	**29.3**	20.0	20.3	20.0	**20.1**
	1.4	a	29	31	30		19	20	20	
		b	29	32	32		19	21	18	
		c	28	31	30		19	19	18	
		Average	28.7	31.3	30.7	**30.2**	19.0	20.0	18.7	**19.2**
	2.8	a	34	33	34		20	23	22	
		b	34	32	35		21	22	21	
		c	33	34	35		22	24	20	
		Average	33.7	33.0	34.7	**33.8**	21.0	23.0	21.0	**21.7**
	5.6	a	35	38	37		24	23	23	
		b	36	37	36		26	25	24	
		c	34	38	36		24	25	24	
		Average	35.0	37.7	36.3	**36.3**	24.7	24.3	23.7	**24.2**
Check (dist. water)		1	6	6	6		6	6	6	
		2	6	6	6		6	6	6	
		3	6	6	6		6	6	6	
		Average	6.0	6.0	6.0	**6.0**	6.0	6.0	6.0	**6.0**

[1]) The filter-paper discs used had a diameter of 6 mm, and all dimensions are the gross transverse measurements of the whole of the clear zone. When the diameter is indicated as 6 mm there is no clear zone round the paper disc and, consequently, the substance (concentration) in question has no antibiotic effect.

described, small discs of stem of about 3–4 mm length were cut from the stems at about 3 cm intervals, i. e. altogether 4 discs (No. 1 = the lowermost one and No. 4 = the uppermost one) from each stem. In deference of the risk of transferring antibiotics (or bacteria, etc.) from one cut to another by means of the knife or the like, an entirely new, sterile razor blade was used for each cut. The discs were immediately placed in numerical order on agar plates (PDA in 10 × 1 cm Petri dishes) which, as far as the tested chemicals and the check were concerned, had just been inoculated with a bacterial suspension of *Ps. caryophylli*. The discs of stem from the cuttings which had been left in the bacterial suspension were placed on sterile agar plates. Then all the dishes were incubated at 28°C, and after 24 hours the diameters of the clear zones around the discs of stem were measured. For the test with the absorption of the bacteria, a record was made of the discs at which growth of *Ps. caryophylli* could be observed.

The results are listed in Table IV, from which it appears that in the course of 24 hours the carnation cuttings are unable to absorb 0.1⁰/₀₀ streptomycin and 0.5⁰/₀₀ 8-hydroxy-quinoline sulphate in sufficient quantities to produce an antibiotic effect; nor had the discs of stem from the check any antibiotic effect of its own.

This negative result was later on investigated in connection with other absorption tests, but the repeated tests showed that pure streptomycin could not be absorbed by the cuttings at all, while 8-hydroxy-quinoline sulphate could be absorbed in some degree after 2 days' treatment. (See Table V, p. 59).

On the other hand, terramycin could be absorbed, even into the uppermost (No. 4) discs of stem, i. e. in the immediate vicinity of the point of growth of the cuttings, but the antibiotic effect, which manifested itself by a clear zone of about 13–14 mm diameter, of which the discs of stem occupied about 4–7 mm, was not so good by far as that of the Agri-mycin, which, around all four discs of stem, produced clear zones with diameters of 23–27 mm (Fig. 13). For the sake of completeness, it should be added that the discs from the check plants did not produce any clear zones around them, and that around the discs of stem originating from the cuttings which had been left in the bacterial suspension, bacterial colonies of *Ps. caryophylli* were constantly being developed at the first (lowermost) disc and also at the second one (No. 2); this means that the bacteria had penetrated 3–4 cm and 6–7 cm, respectively, up the stems; only in a single case was bacterial growth of *Ps. caryophylli* found at the third disc, i. e. about 9 cm up the stem!

Table IV. Ability of carnation cuttings, "Petersen's Pink Sim", to absorb antibiotics, 8-hydroxy-quinoline sulphate, and *Pseudomonas caryophylli* in 24 hours at 18–22° C.

Name	Concentration ‰	Stem disc No.	Diameter of clear zone in mm[1]) Cutting No. 1	2	3	4	Average
Agri-mycin 100	5.6	1	29	29	27	25	**27.5**
		2	26	24	26	24	**25.0**
		3	27	24	25	24	**25.0**
		4	28	22	24	19	**23.3**
Streptomycin	0.1	1			—	—	—
		2	—	—		—	—
		3	—	—	—	—	-
		4	-			—	—
Terramycin	0.1	1	15	14	15	11	**13.8**
		2	14	12	16	12	**13.5**
		3	14	15	15	14	**14.5**
		4	13	12	14	13	**13.0**
8-hydroxy-quinoline sulphate	0.5	1	—		—	—	—
		2	—	—	—	—	—
		3	—	—	—	—	—
		4	—	—	—	—	—
Check (distilled water)		1	—	—	—	—	—
		2	—	—	—	—	—
		3	—	—	—	—	—
		4	—	—	—	—	—
Pseudomonas caryophylli		1	+	+	+	+	
		2	+	+	+	+	
		3	O	O	+	O	
		4	O	O	O	O	

[1]) The dimensions are the diameters of the entire clear zone, irrespective of the discs-of-stem diameters at about 4–6 mm.

— no antibiotic effect (clear zone) observed.
O no bacterial growth observed.
+ bacterial colonies of *Ps. caryophylli* observed. Stem discs No. 1 = 3 cm up the stem; No. 2 = 6 cm, No. 3 = 9 cm, and No. 4 = 12 cm up the stem.

iii. Ability of carnation stems to absorb various aqueous solutions of Agri-mycin 100 in 12, 24, and 48 hours. The studies of the antibiotic effect of the various substances thus seem to indicate, both in the tests with the discs of filter-paper and, particularly, in the absorption tests with carnation cuttings, that Agri-mycin 100 is superior

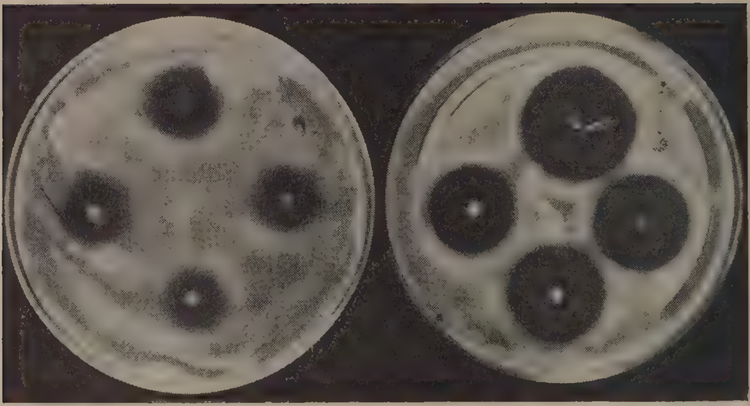

Fig. 13. Antibiotic effect around discs of stem of "Petersen's Pink Sim" placed on PDA sown with a pure culture of *Ps. caryophylli*. Left: From a plant which had absorbed terramycin ($0.1^0/_{00}$ = 100 ppm). Right: From a plant which had absorbed Agri-mycin 100 ($5.6^0/_{00}$ = 80 ppm in respect of content of terramycin, i. e. a slightly weaker concentration). Uppermost disc in the dishes ("at 12 o'clock") = No. 1; disc No. 2 at "3 o'clock", etc. Photographed after 2 days. ×0.5.

to the other substances tested in respect of antibiotic effect. Consequently, the continued tests, in which attempts were made at finding a method and an agent for systemic control of bacterial stem-crack in carnations by means of antibiotics, were then particularly devoted to Agri-mycin 100.

In the absorption tests so far carried out, an absorption time of 24 hours had been used as the starting point, for one thing with a view to the possible adoption of a combined treatment with a rooting stimulant, but as the use of Agri-mycin over such a long time seemed to have a very powerful effect, it was tried whether it would be possible, by using a shorter time of treatment or weaker concentrations, to attain as favourable an antibiotic effect, or at least a definite antibiotic effect.

In order to have these questions elucidated, another absorption test was made, in which the ability of the cuttings to absorb Agri-mycin 100 of 4 different concentrations (0.7, 1.4, 2.8, and $5.6^0/_{00}$) in 12, 24, and 48 hours, respectively, and at 18–22° C, was investigated. As a check, partly sterile, distilled water and partly an $0.5^0/_{00}$ aqueous solution of 8-hydroxy-quinoline sulphate were included. The test was carried out in a manner similar to the one described above, and again 10 cuttings of the variety "Petersen's Pink Sim" were used per beaker (concentration), or altogether 60 cuttings.

After 12 hours, 3 cuttings were selected from each of the 6 lots, and from each of these cuttings 4 discs of stem were cut in the usual way. The discs were immediately placed on PDA inoculated with *Ps. caryophylli.* This selection of 3 cuttings per lot was repeated after 24 as well as after 48 hours. All the dishes were incubated at 28°C. The results of the test are listed in Table V.

Table V. Ability of carnation cuttings, "Petersen's Pink Sim", to absorb Agri-mycin 100 and 8-hydroxy-quinoline sulphate in 12, 24, and 48 hours, respectively, at 18–22°C.

Name	Concentration ‰	Stem disc No.	Diameter of clear zone in mm[1]) 12 hours Cutting No. 1	2	3	Average	24 hours Cutting No. 1	2	3	Average	48 hours Cutting No. 1	2	3	Average
Agri-mycin 100	5.6	1	15	14	15	**14.7**	26	21	19	**22.0**	22	20	20	**20.7**
		2	10	7	14	**10.3**	22	21	17	**20.0**	20	20	20	**20.0**
		3	—	—	9	**3.0**	15	21	8	**14.7**	20	19	18	**19.0**
		4	—	—	6	**2.0**	9	7	6	**7.3**	18	15	10	**14.3**
Agri-mycin 100	2.8	1	—	14	13	**9.0**	6	12	9	**9.0**	15	17	20	**17.3**
		2	—	—	—	—		—	—	—	15	11	15	**13.7**
		3		—	—	—	-	·	-		15	10	16	**13.7**
		4	—	—	—	—	—	—	—	—	15	8	17	**13.3**
Agri-mycin 100	1.4	1	—	—	—	—	—	—	—	—	16	12	12	**13.3**
		2	—	—	—	—	—	—	—	—	14	12	11	**12.3**
		3	—	—	—	—	—	—	—	—	12	10	9	**10.3**
		4	—	—	—	—	—	—	—	—	13	10	—	**7.7**
Agri-mycin 100	0.7	1	—	—	—	—	—	—	—	—	7	10	7	**8.0**
		2	—	—	—	—	—	—	—	—	7	8	10	**8.3**
		3	—	—	—	—	—	—	—	—	6	8	9	**7.7**
		4	—	—	—	—	—	—	—	—	—	9	6	**5.0**
8-hydroxy-quinoline sulphate	0.5	1	—	—	—	—	—	—	—	—	30	19	5	**18.0**
		2	—	—	—	—	—	—	—	—	19	15	—	**11.3**
		3	—	—	—	—	—	—	—	—	16	—	—	**5.3**
		4	—	—	—	—	—	—	—	—	—	—	—	—
Check dist. water		1	—	—	—	—	—	—		—	—			—
		2	—	—	—	—	—	—	—	—	—	—		
		4					—	—	—			—	—	
		4	—	—	—	—			—	—			—	—

[1]) The dimensions are the diameters of the entire clear zone, irrespective of the diameter of about 4–6 mm of the discs of stem.

From Table V it will be seen that, in the circumstances 12 hours seem to be too short a time of treatment. Only the lowermost two discs of stem (Nos. 1 and 2) from the cuttings of the lot which had been left in $5.6^{0}/_{00}$ Agri-mycin displayed a fairly definite, although weak, antibiotic effect. In the cuttings which had been left in $2.8^{0}/_{00}$ Agri-mycin a weak and uncertain antibiotic effect was found for the first (lowermost) disc of stem, while for the other discs from these cuttings no effect was found at all. The samples from the cuttings that had been left in $1.4^{0}/_{00}$ and $0.7^{0}/_{00}$ Agri-mycin and in $0.5^{0}/_{00}$ 8-hydroxy-quinoline sulphate, as well as those from the check plants, likewise showed no effect at all.

The next "stem samples" were taken out after 24 hours' absorption of the different liquids. Apart from the fact that the antibiotic effect ascertained in the discs from the cuttings which had been left in $5.6^{0}/_{00}$ Agri-mycin was now found to be stronger and more definite in all 4 discs of stem, the cuttings from the other concentrations did not seem to have been "impregnated" to any appreciable greater degree after 24 hours than after 12 hours. The antibiotic effect radiating from the lowermost (No. 1) disc of the cuttings which had been left in $2.8^{0}/_{00}$ Agri-mycin seemed still very weak, though a little more certain, but the other samples included in the test were constantly found to have no antibiotic effect.

A better result was obtained after the plants had been left for 48 hours in the different liquids. Following the treatment with $0.5^{0}/_{00}$ 8-hydroxy-quinoline sulphate, $0.7^{0}/_{00}$ and $1.4^{0}/_{00}$ Agri-mycin, some—although weak and uncertain—antibiotic effect was ascertained. This effect was especially found to be uncertain in the uppermost (No. 4) and next-uppermost (No. 3) discs. All the discs of stem from the cuttings which had been left in $2.8^{0}/_{00}$ Agri-mycin now displayed a more definite antibiotic effect. Also in the two uppermost discs (Nos. 3 & 4) had this effect improved rather considerably in the cuttings from the lot which had been left in $5.6^{0}/_{00}$ concentration.

The test was repeated later, with almost the same result, and it thus seems fairly certain that, with an absorption time of at least 24 hours, it is highly probable that with a $5.6^{0}/_{00}$ Agri-mycin 100 solution so much antibiotic can be taken up into the carnation cuttings that even the uppermost parts of the stem have a greatly inhibiting effect on the growth of *Ps. caryophylli*, and if the absorption time is extended to 48 hours, the antibiotic effect becomes very definite. If, however, this rather strong concentration is reduced to $2.8^{0}/_{00}$, it will be necessary to allow the plants to stand in the substance for at least 2 days so that the whole

stem of the cutting may be "impregnated" by the Agri-mycin. It should be noted, however, that although $2.8^{0}/_{00}$ Agri-mycin will reach the top of the cuttings in 48 hours, the antibiotic effect found for the uppermost stem-portions is a good deal less than when the $5.6^{0}/_{00}$ solution is used.

The effect of $1.4^{0}/_{00}$ and $0.7^{0}/_{00}$ Agri-mycin solution is, in general, so weak that a definite antibiotic effect in the top of the cuttings cannot be counted on.

Concurrently with the investigation of the ability of the stems to absorb Agri-mycin, tests were made to see whether the presence of this antibiotic could be demonstrated in the leaves also. However, even in the cuttings which had been standing for 24 hours in a $5.6^{0}/_{00}$ Agri-mycin solution and whose stems—at any rate as far as the lower half was concerned—had constantly displayed a definite antibiotic effect, no effect was found in transverse sections cut from the base of the lowermost two leaf-pairs of the cutting. The investigations were later repeated several times, "random tests" on the ability of the leaves to absorb Agri-mycin being made now and then simultaneously with the other absorption tests, but attempts at demonstrating any antibiotic effect in the basal parts of carnation leaves were all abortive.

As the tests described above seemed to hold out the hope of an effective control of *Pseudomonas caryophylli* by the use of either $2.8^{0}/_{00}$ or $5.6^{0}/_{00}$ aqueous solutions of Agri-mycin 100 absorbed by the cuttings, a large number of absorption tests were then made on carnation cuttings of different varieties with the usual concentrations of Agri-mycin 100. It was, however, important—with a view to the previously mentioned possibility of a combined treatment with a rooting stimulant and an antibiotic—to keep the time of treatment under 24 hours. So, in the next absorption tests the absorption time was limited to 24 hours. Otherwise, the tests were carried out under the same conditions and in the same way as the previous tests, but this time each test comprised 5 cuttings, as against 3 in the preceding tests. The results of 4 tests, chosen at random, are listed in Table VI and show clearly that in 24 hours the absorption by the cuttings of a $2.8^{0}/_{00}$ aqueous solution of Agri-mycin 100 is so inadequate that an effective control of bacteria which are found only a few centimetres up the stems can absolutely not be reckoned on. However, the experiments tended to indicate that neither is even a $5.6^{0}/_{00}$ solution of Agri-mycin 100 with certainty taken right up to the top in all cuttings. Thus, in several cases no antibiotic effect was found in the uppermost or even in the next uppermost discs of stem. However, the effect of $5.6^{0}/_{00}$ Agri-mycin was largely so good that this

Table VI. The ability of carnation cuttings, "Petersen's Pink Sim", to absorb Agri-mycin 100 in different concentrations in 24 hours and at 18–22°C.

Name	Concentration ‰	Stem disc No.	Lot No. I Cutting No. 1	2	3	4	5	Average	Lot No. II Cutting No. 1	2	3	4	5	Average	Lot No. III Cutting No. 1	2	3	4	5	Average	Lot No. IV Cutting No. 1	2	3	4	5	Average	Total average
			Diameter of clear zone in mm[1])																								
Agri-mycin 100	5.6	1	18	10	17	17	13	**15.0**	20	21	17	20	18	**19.2**	16	15	17	14	16	**15.6**	17	26	19	17	19	19.6	**17.4**
		2	6	—	18	18	—	**8.4**	19	19	18	20	12	**17.6**	15	15	17	16	16	**15.8**	17	20	18	16	20	18.2	**15.0**
		3	—	—	9	12		**4.2**	12	18	7	15		**10.4**	9	15	18	15	11	**13.6**	16	12	18	8	18	14.4	**10.7**
		4	—	—	—	—	—		—	11	—	6	—	**3.4**	—	12	7	14	—	**6.6**	8	—	18	—	13	7.8	**4.5**
Agri-mycin 100	2.8	1	19	14	16	15	14	**15.0**		14	12	10	18	**10.8**	14	12	—	12	8	**9.2**	12	11	17	—	14	10.8	**11.5**
		2	12	13	9	15	13	**12.4**	—	—	—		7	**1.4**	15					**3.0**	—						**4.2**
		3	—	—	—	—	6	**1.2**		—		—	—		13	—	—	—	—	**2.6**	—	—	—	—	—	—	**1.0**
		4	—	—	—	—	—			—	—	—		—	7	—	—	—	—	**1.4**	—	—	—	—	—	—	**0.4**
Agri-mycin 100	1.4	1	—	9	12	13	—	**6.8**	15	17	11	15	8	**13.2**	11	15	13	—	—	**7.8**	9	—	10	15	13	9.4	**9.3**
		2	—	—	—	—	—				—	7	—	**1.4**	—	—	—	—	—	—	—	—	—	—	10	2.0	**0.9**
		3	—	—			—	—	—	—	—	—	—			—	—	—	—		—		—		—	—	—
		4	—			—	—			—		—	—		—	—		—	—	—	—	—		—	—	—	—
Agri-mycin 100	0.7	1	—	—	9			**1.8**	6		—			**1.2**	12	12	—	11	9	**8.8**	—	—	12			2.4	**3.6**
		2						—						—	—					—	—	—	—		—	—	—
		3	—	—						—		—	—						—	—	—	—	—	—	—	—	
		4	—	—	—	—	—		—	—	—				—	—	—	—	—		—	—	—	—		—	—
Check (dist. water)		1	—	—						—	—			—		—	—	—	—				—				—
		2					—	—	—	—							—	—			—	—	—	—	—	—	—
		3	—	—					—	—	—	—						—	—	—	—	—	—	—	—	—	—
		4	—	—		—	—	—	—	—	—				—	—	—	—	—		—	—	—	—	—	—	—

1) The dimensions are the diameters of the entire clear zones, irrespective of the diameter of 4–6 mm of the discs of stem.
— denotes that no antibiotic effect (clear zone) has been observed outside the discs of stem.

concentration must be considered a good starting-point for the continued control experiments, provided the cuttings were able to stand this concentration at all.

iv. Absorption and distribution of Agri-mycin 100 in stems of carnation cuttings. In connection with the described absorption tests the question naturally arose whether the bacteria in the diseased vessels could really be expected to be killed either by the direct absorption of the antibiotic by the bacteria-filled vessels or by the diffusion of it from the healthy vessels to the diseased ones.

Tests were therefore made to find out whether it was possible to demonstrate that the Agri-mycin diffused transversely in the stems of cuttings that had been left in a $5.6^{0}/_{00}$ Agri-mycin solution for 24 hours. For these tests some ten well-developed cuttings of the variety "White Sim" were selected. The stems of these cuttings were generally about 6–8 mm in diameter. The investigation was commenced so that a 4 cm long piece of stem was cut off from the middle of the treated cuttings. From each end of this stem-piece a 2–3 mm thick disc was then cut off and immediately placed on agar inoculated with *Ps. caryophylli*. In this way it was checked whether the antibiotic had been absorbed throughout the length of the stem-pieces cut off. Without awaiting the result of this test, which, incidentally, proved that all the stem-pieces were actually completely saturated with antibiotics, all the pieces were immediately subjected to the further investigation the aim of which was to establish whether the antibiotic absorbed could be found in, or just under, the epidermis, and whether it was found also in the pith of the stems. With this object in view, each of the 10 stem-pieces was subdivided into 4. One of these, which was not subjected to further treatment, was placed direct on an agar surface (PDA) inoculated with *Ps. caryophylli*, the unwounded epidermis of the stem-piece touching the agar. Another quarter of the stem-piece was likewise placed on agar, but the epidermis of this quarter was first scraped off with a knife in an about 2 mm wide stripe in the full length of the piece, after which the wound thus produced was placed in contact with the agar surface. The third quarter was just placed on the medium with its end face downward.

From the pith of the fourth fraction a small prismatic piece was cut out and, finally, placed on agar sown with bacteria. These "pith-prisms" were produced so that the whole of the outside tissue with epidermis and vascular tissue was removed by 4 longitudinal cuts. To prevent, as far as possible, the transfer of antibiotic from one cut surface to another,

each individual cut was made with a new, sterile razor blade. In this way the size of the piece of pith was reduced on all 4 sides by another 3 or 4 peelings until, finally, the "pith-prism" measured a little more than 1 mm in cross-section and was about 3–4 mm long. This small piece was halved transversely, and both halves were then placed on PDA, the cut surfaces produced by the halving facing the agar. The test comprised 10 cuttings and altogether 20 Petri dishes, 4 stem-pieces from each cutting being placed in the same dish, while the 2 check-discs cut off at the beginning of the experiment were placed in dishes of their own, i. e. altogether 2 dishes per cutting. As usual, all the dishes were left in an incubator at 28°C for 24 hours, after which all the stem-pieces appeared to contain a sufficient amount of antibiotic throughout their length to produce distinct, clear zones around both end-discs (the check-discs). The test further showed that from all surfaces of wounds—whether these were transverse or longitudinal—distinct, clear zones of a diameter of about 15–20 mm had formed. Only from the stem-pieces whose unwounded epidermis touched the agar surface had no antibiotic penetrated into the agar. From the end faces of these horizontal stem-pieces a small, crescent-shaped, clear zone was, however, visible, presumably originating from antibiotic-containing sap that had run on to the agar surface.

The tests thus seem to indicate that Agri-mycin is able to diffuse from the vessels both to epidermis and into the pith, and so, after the treatment, the stems must be considered to have been saturated with the substance.

Part of the test was continued, the stem-pieces, about 1 cm long, which so far had been standing on the agar in an upright position, being halved after 24 hours and transferred to new Petri dishes containing PDA inoculated with bacteria. These halved pieces were placed in such a way that the newly formed cut-surfaces were now brought in touch with the medium. The halving and transfer to fresh agar were repeated altogether 3 times, so the last discs were only $\frac{1}{8}$ the original length, or about 1 mm thick. Around all the discs practically equally large, clear zones appeared, which agreed excellently with the figures listed in Table VI under $5.6^0/_{00}$. Thus, Agri-mycin seems not only to make its way into all parts of the stems but also to be retained so firmly in the tissues that, at any rate, the agar medium is unable to extract so much antibiotic, even from thin discs of stem, that smaller parts of such discs give a reduced antibiotic reaction when placed on agar again. Altogether, it would appear that the portion of the antibiotic substance which is absorbed by the agar medium originates solely from the content of the cells which had been cut to pieces.

v. Keeping qualities of Agri-mycin 100 in carnation cuttings. The problem of the keeping qualities of Agri-mycin in carnation cuttings being an important one, new absorption tests were made, in which cuttings of the variety "Petersen's Pink Sim" were, as an initial measure, left for absorption in a $5.6^0/_{00}$ aqueous solution of Agri-mycin 100. After 24 hours at 20–22°C, the cuttings were taken out of the liquid and rinsed thoroughly under a water tap, after which they were placed in new beakers with clean tap-water. The water was renewed every 24 hours and, in addition, 3 plants were selected, out of which 4 discs of stem were cut, as usual, for placing on PDA sown with *Ps. caryophylli.* The taking of samples was done daily for a week, but after 7 days it was still impossible to demonstrate any reduction in the antibiotic effect of the discs of stem on *Ps. caryophylli.* By way of example, it may be mentioned that the total average figures for the diameters of the clear zones produced around the 12 discs of stem which were examined every 24 hours, were calculated to be 15, 15, 15, 13, 20, and 19 mm, respectively, for the first 6 days, i. e. an effect which agreed excellently with the results obtained so far with the absorption of $5.6^0/_{00}$ Agri-mycin (compare Table V, p. 59). It was considered interesting, however, to ascertain whether the antibiotic effect could be maintained even longer, and a prolonged investigation of the keeping qualities of Agri-mycin, partly in carnation stems and partly as a liquid stored in a bottle, was commenced. Once more the cuttings were treated for 24 hours, after which the Agri-mycin solution was replaced with pure water, and during the following 4 weeks the water in the beakers was renewed each day. Sampling was done each week, the keeping qualities of the pure liquids being tested by the soaking of discs of filter-paper and placing of them on agar sown with *Ps. caryophylli*, while the keeping qualities of the Agri-mycin in the cuttings were tested once more by the usual cutting-out of discs of stem and placing them on agar also inoculated with bacteria. The extent of the clear zones that appeared both around the discs of paper and the discs of stem indicated plainly that the effect of the substance in the cuttings as well as in the pure liquid was not diminished in the course of 4 weeks at a temperature of about 18–22°C.

Accordingly, judging from all appearances, carnation cuttings are able to take up certain concentrations of Agri-mycin 100—particularly 2.8 and $5.6^0/_{00}$—in 24 hours. This substance, again, is able to spread so uniformly in the stems that all parts of them will most frequently have a good antibiotic effect on *Ps. caryophylli*, and it looks as if this effect will remain unchanged in the stems for 4 weeks or perhaps even longer.

Table VII. Number of carnation cuttings of the variety "White Sim" which, on the propagation bed, had

Concentration	Not treated with a "rooting stimulant"							
	Streptomycin		Terramycin		Streptomycin + terramycin		Check	
	Roots		Roots		Roots		Roots	
	None	Normal	None	Normal	None	Normal	None	Normal
0.1‰ (= 100 ppm)	50	0	50	0	50	0	0	50
0.01‰ (= 10 ppm)	1	49	0	50	1	49	0	50
0.001‰ (= 1 ppm)	0	50	0	50	0	50	0	50

vi. Reaction of carnation cuttings to treatment with Agri-mycin 100, streptomycin, and terramycin. Now, the main question was whether the cuttings could stand the treatment, and tests were therefore made on the treatment of various carnation varieties, e. g. "Petersen's Pink Sim", "White Sim", "Galazzo Sim", and "Lucky Strike", with 5.6 and 2.8‰ Agri-mycin; 100 cuttings of each variety were used per concentration together with 100 check plants, i. e. 900 cuttings in all. The check cuttings were left in sterile distilled water. The time of treatment was limited to 24 hours, and the cuttings were then rooted in a carnation nursery under exactly the same conditions as applied to the other carnation cuttings in the nursery. The cuttings were left to stand for 3 weeks, but during this period nothing unusual was noticed in the appearance of the treated cuttings. During the last 5 or 6 days of the period it looked, however, as if the hitherto fresh, greenish-blue colour of the plants assumed a faint, greyish hue. After 3 weeks, all the test plants were taken out of the sand, and it was then observed that not a single one of the treated cuttings had formed roots, while all the check plants had produced a normal and well developed root system. The experiment was repeated immediately, but the result was the same.

The failure of these cuttings to form roots must, unfortunately, be ascribed solely to the treatment with Agri-mycin 100, since hundreds of the cuttings of the same varieties, in the same period and on the same propagation bench, produced perfectly normal roots. The question was, however, whether it was the comparatively strong concentrations or whether the Agri-mycin contained certain "impurities" which had so great a toxic effect on the carnations that the ability to form roots was completely lost.

after 24 hours' absorption of solutions of pure antibiotics, and after 5 weeks formed normal roots or none.

Treated with a "rooting stimulant"							
Streptomycin		Terramycin		Streptomycin + terramycin		Check	
Roots		Roots		Roots		Roots	
None	Normal	None	Normal	None	Normal	None	Normal
50	0	50	0	50	0	1	49
0	50	2	48	0	50	0	50
0	50	0	50	0	50	1	49

To throw light on this question, another lot of cuttings of the variety "White Sim" was treated, partly with solutions of pure streptomycin and terramycin, respectively, and partly with a mixture of these two antibiotics in the same proportion as in Agri-mycin 100 (i. e. $15\,^{0}/_{0}$ streptomycin and $1.5\,^{0}/_{0}$ terramycin). Finally, the said 3 solutions were used in 3 different concentrations, corresponding to $0.1\,^{0}/_{00}$ (= 100 ppm), $0.01\,^{0}/_{00}$ (= 10 ppm), and $0.001\,^{0}/_{00}$ (= 1 ppm). The test was carried out with altogether 100 cuttings per antibiotic per concentration, as well as 100 untreated cuttings as a check, i. e. $400 \times 3 = 1200$ cuttings in all. After the treatment, half of the plants were dusted on the cut-surfaces with "Floramon", a rooting stimulant, while the other half remained untreated. In order to allow the cuttings sufficient time to form roots, the experiment was not concluded until more than 5 weeks had passed.

The results of the experiment are listed in Table VII, from which it also appears that a concentration of $0.1\,^{0}/_{00}$ (= 100 ppm) of the different antibiotics—corresponding to the concentration of streptomycin in $0.7\,^{0}/_{00}$ Agri-mycin solution—was so toxic to carnation cuttings that their ability to form roots was completely lost, whether they were treated with pure antibiotics (streptomycin or terramycin) alone or with a mixture of these two antibiotics. If, however, the concentration of the various antibiotics was reduced to $0.01\,^{0}/_{00}$ (= 10 ppm) or to $0.001\,^{0}/_{00}$ (= 1 ppm), none of the antibiotics used prevent the formation of normal roots. In the tests with the low concentrations it was thus impossible to ascertain any difference in the root systems of the check plants and the treated cuttings, and in most cases the colour of the leaves was also perfectly normal; but in the plants that had been treated with 10 ppm terramycin a faint, greyish discoloration of the leaves developed in the course of the last 10 days or so before they were taken out of the sand.

This might indicate that even this weak concentration of terramycin had some toxic effect on the carnations.

When pure antibiotics were used in a concentration of $0.1^0/_{00}$ (= 100 ppm), a considerable toxic effect on the carnations was also found, and the detrimental effect of Agri-mycin could then hardly be due to the occurrence of any impurities in the preparation, but presumably to too strong a concentration of this antibiotic.

Attempts were made at verifying the correctness of this hypothesis, by allowing carnation cuttings of the varieties "White Sim" and "Petersen's Pink Sim" to absorb Agri-mycin solutions of the concentrations 5.6, 0.7, 0.33, 0.27, 0.20, 0.13, and $0.07^0/_{00}$. 50 cuttings of each variety were used per concentration, together with 100 check plants, i. e. 450 × 2 = 900 cuttings in all. When, after more than 6 weeks, they were removed from the propagation bed, it appeared that even a concentration of $0.07^0/_{00}$ Agri-mycin—corresponding to 10 ppm streptomycin—had a considerable, toxic effect on the development of roots on the cuttings. A new absorption test was therefore carried out, with still lower concentrations of Agri-mycin, viz. 0.13, 0.07, 0.04, and $0.007^0/_{00}$, but for this experiment plants of the varieties hitherto used were, unfortunately, unobtainable. The variety "Galazzo Sim" was then used instead—100 plants per concentration plus 100 untreated check plants, i. e. 500 cuttings in all. The experiment was carried out under the same conditions as the preceding one, and the results of both experiments are listed in Table VIII, from which it appears, partly that even concentrations as low as 0.07 and $0.007^0/_{00}$ Agri-mycin 100 have a considerable retarding effect on the rooting of carnation cuttings, and partly that the carnation varieties display a great difference in tolerance to the antibiotics used. Thus, "White Sim" was considerably less resistent than both "Petersen's Pink Sim" and "Galazzo Sim".

The plants which survived the treatment in the last-mentioned test (Table VIII) were, on conclusion of the test, planted out partly in beds and partly in pots and kept under observation for more than 6 months, but in that period nothing abnormal was apparent in the plants.

vii. Reaction of rooted cuttings to dipping of roots in Agri-mycin 100. The ability of rooted cuttings to take up Agri-mycin 100 through the roots was investigated by placing 2 × 15 young (about 5-week-old) plants of each of the varieties "White Sim" and "Petersen's Pink Sim" in beakers with 200 ml $0.5^0/_{00}$ and $1.0^0/_{00}$ aqueous solutions of Agri-mycin 100, respectively. The plants were left in a light laboratory

Table VIII. Formation of roots in carnation cuttings of 3 different varieties after 24 hours' treatment with different concentrations of Agri-mycin 100.

Concentration of Agri-mycin 100 in °/₀₀. In brackets corresponding conc. of streptomycin, in ppm.	Number of cuttings with none, few or many (= normal) roots, respectively								
	"White Sim"			"Petersen's Pink Sim"			"Galazzo Sim"		
	None	Few	Many	None	Few	Many	None	Few	Many
5.60 (800 ppm)	50	0	0	50	0	0			
0.70 (100 ppm)	50	0	0	18	0	32			
0.33 (50 ppm)	48	2	0	7	10	33			
0.27 (40 ppm)	49	1	0	8	0	42			
0.20 (30 ppm)	48	0	2	1	1	48			
0.13 (20 ppm)	46	2	2	2	0	48	4	32	64
0.07 (10 ppm)	14	1	35	4	1	45	8	17	75
0.04 (5 ppm)							4	8	88
0.007 (1 ppm)							8	6	86
Check, distilled water	1	0	99	0	0	100	2	0	98

at about 18–22°C. After 3 days, 5 plants from each of the four test links were selected, after which, as usual, discs of stem were cut out—the first about 3 cm from the base of the stem—and placed on PDA inoculated with bacteria, but in no case could antibiotic effect from the lowermost stem-parts be demonstrated, even not in new samples taken after altogether 5 days. Furthermore, the leaves of the plants which had been left in the liquids for more than a week were seen to assume a greyish colour, and after about a fortnight, all the plants had withered completely. Some check plants, which had been left in distilled water, were undamaged after the same period.

Consequently, in the present circumstances Agri-mycin 100 could not be absorbed through the roots of the young carnation plants; nor could there be any doubt that Agri-mycin had a more toxic effect on rooted cuttings than on rootless cuttings.

viii. Conclusions. The experiments made so far, in which it was tried to let rootless carnation cuttings absorb different solutions of Agri-mycin 100 or pure streptomycin and terramycin, respectively, in the hope of being able in that way to kill bacteria in the vessels of the cuttings, show that *Pseudomonas caryophylli* is greatly susceptible to Agri-mycin

100 as well as to both streptomycin and terramycin, and that especially Agri-mycin 100 is relatively readily taken into the carnation stems, where it even makes its way into all the cells of the stem, preserving an antibiotic effect for several weeks. The possibility of carrying through a systemic control of *Ps. caryophylli* by means of antibiotic-containing liquids thus seems to exist. On the other hand, the great susceptibility of carnations to the antibiotics so far used is an essential obstacle to the attainment of applicable results through this means. It should be borne in mind that the concentrations which could be used for the carnations should preferably not exceed 0.007–0.07$^0/_{00}$—though for certain varieties it might be as high as 0.13$^0/_{00}$— Agri-mycin (i. e. 1, 10 and 20 ppm, respectively). However, since solutions with about 10 times as large a content of antibiotics as the maximum concentrations just mentioned have proved to be insufficient to produce a definite antibiotic effect—if any at all—in the uppermost stem parts of carnation cuttings, and since weak solutions have often only a bacteriostatic effect, the author is of the opinion that neither Agri-mycin 100 nor its components are of any practical value in the control of bacterial stem-crack of carnation.

B. Indirect Control Measures

a. Introduction

Concurrently with the author's efforts to control bacterial stem-crack of carnation by means of chemicals, experiments were being made which aimed at finding a method of separating all diseased cuttings from the healthy ones immediately after the breaking-off of the cuttings. Such sorting, if reliable, would offer several decisive advantages, seeing that the healthy cuttings could then be placed together on the propagation bed without any risk of infection, and also the use of chemicals would be superfluous. Furthermore, a really effective, early sorting-out of the diseased cuttings must be assumed to lead to a relatively quick elimination of the disease. This indirect control would therefore be particularly desirable, both on cultivating and plant pathological grounds, but as bacterial stem-crack of carnation does not produce outward symptoms in newly picked cuttings, because at this stage of the life of the plant the bacteria are most frequently present only in a few, not yet discoloured, vessels of the stems, the sorting merely by subjecting the cutting to a careful exterior examination is impracticable. On the other hand, an examination of the interior of the stem will in the majority of cases disclose the disease.

As far as the author is able to judge, examination of the interior of a carnation stem can be carried out in two fundamentally different ways. One method is to ascertain by watching thin cross sections of stem of the cuttings under the microscope, whether the cross section has a normal, healthy appearance or whether, in consequence of the action of pathogenic bacteria, any discolouring or deformation of the vascular tissues can be found. The other method of examination is based on biological testing of the interior sterility of the carnation stems and is carried out in practice by the placing of small pieces of superficially sterilized carnation stems either on an agar medium—usually PDA—or in water, which is later on streaked on to PDA in Petri dishes—or the placing of the stem-pieces in nutrient broth in test tubes. In any case, a certain number of days will pass before it is possible to ascertain whether the stem-pieces are sterile—in which case the cutting in question is then declared to be healthy—, or whether the cutting is attacked by a bacterial disease indicated by the growth of pathogenic bacteria around the stem-pieces.

b. The "Microscopy Method"

At first glance, the examination of cross sections of stem under the microscope appears to be a very quick and easily practicable method, seeing that in the majority of cases it is then possible to decide at once whether the cutting in question is healthy or diseased, and it is thus unnecessary for the cuttings to stand for several days pending the result of the test, as is necessary in the biological tests. It was therefore natural to try and apply this method when "bacterial stem-crack" appeared in Denmark for the first time, and the grower in question was anxious to obtain as quickly as possible a few hundred healthy cuttings for the further propagation.

In practice, however, the "microscopy method" appeared not to be as quick as expected, partly because it was found necessary to examine at least 3 cross sections[1]) to ensure a reliable assessment of the cross sections of stem, and partly because the actual examination of the cross sections took so much time that two persons between them were only able to check about 200 cuttings in 8 hours, which proved later on to be less than half the number of cuttings which could be checked daily by the biological methods. Finally, it was found that the microscopic

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[1]) In the routine it was difficult at once to cut off a whole disc of stem of a suitable thickness; as a substitute, 3–4 smaller slices of the stem were therefore used.

Table IX. The "Method Test". A survey of different testing results for 4 lots (I, II, methods: The "microscopy method" ("M"); the "agar method" ("Agar"); isolation by spreading of an aqueous

Cutting No.	Lot No. I				Lot No. II			
	"M"	"Agar"	"Broth"	"S"	"M"	"Agar"	"Broth"	"S"
1	+	+	+	+	+	+	+	+
2	–	(+)	(+)	(+)	?	(+)	(+)	(+)
3	?	(+)	+	+	+	+	+	+
4	+	(+)	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+
6	–	+	+	+	+	+	+	+
7	+	+	+	+	–	(+)	(+)	(+)
8	?	(+)	(+)	(+)	+	+	+	+
9	+	+	+	+	+	+	+	+
10	–	–	+	+	+	+	+	+
11	?	+	+	+	+	+	+	+
12	–	–	(+)	(+)	+	+	+	+
13	+	+	+	+	–	–	+	+
14	+	+	+	+	–	–	–	–
15	+	+	+	+	–	–	(+)	(+)
16	+	+	+	+	?	(+)	(+)	(+)
17	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+
19	–	–	+	+	?	+	+	+
20	+	+	+	+	+	+	+	+
21	–	–	–	–	?	+	+	+
22	?	(+)	(+)	(+)	–	(+)	+	+
23	?	+	+	+	–	–	–	–
24	?	(+)	(+)	(+)	–	(+)	(+)	(+)
25	–	(+)	+	+	–	(+)	(+)	(+)

\+ *Pseudomonas caryophylli* demonstrated.
? brown vessels.

examination was not perfectly reliable either. Now and again, it was possible by the biological methods to isolate *Pseudomonas caryophylli* from cuttings which, by the microscopic examination of the cross sections, had been found to be healthy (Table IX; I-6, I-19, III-7, etc.). To this may be added that neither was it always possible to see clearly what was the cause of certain abnormalities; in particular, it was difficult to decide whether a quite faint, brownish-yellow discoloration of one

III & IV) of 25 cuttings of the variety "Harvest Moon", examined on 4 different the "nutrient broth method" ("Broth"); and the check, the "suspension method", suspension on to PDA ("S").

Lot No. III				Lot No. IV			
"M"	"Agar"	"Broth"	"S"	"M"	"Agar"	"Broth"	"S"
?	(+)	(+)	(+)	–	+	+	+
?	–	–	–	–	(+)	+	+
?	(+)	(+)	(+)	?	(+)	(+)	(+)
+	+	+	+	–	–	–	–
?	+	+	+	–	(+)	(+)	(+)
+	–	+	+	?	–	(+)	(+)
–	+	+	+	–	(+)	(+)	(+)
+	+	+	+	?	(+)	(+)	(+)
–	(+)	(+)	(+)	?	(+)	(+)	(+)
?	(+)	+	+	–	+	+	+
–	–	(+)	(+)	?	+	+	+
–	(+)	(+)	(+)	?	(+)	(+)	(+)
–	(+)	–	–	–	(+)	(+)	(+)
?	–	+	+	–	–	–	–
?	(+)	+	+	?	(+)	(+)	(+)
–	–	–	–	?	(+)	(+)	(+)
?	–	(+)	(+)	?	(+)	(+)	(+)
–	(+)	(+)	(+)	?	–	–	–
?	(+)	(+)	(+)	–	–	(+)	(+)
+	–	+	+	–	(+)	(+)	(+)
–	–	+	+	?	(+)	(+)	(+)
–	(+)	(+)	(+)	–	(+)	(+)	(+)
?	(+)	+	+	–	–	–	–
?	–	+	+	?	(+)	(+)	(+)
–	–	–	–	–	(+)	(+)	(+)

(+) bacterial growth demonstrated (species uncertain).
– healthy cross sections of stem or no bacterial growth on medium.

or more vessels originated from attacks by bacteria or from other causes. It should be observed, for example, that the microscopic examination in December 1953 of a lot of 25 carnation cuttings of the variety "Harvest Moon" disclosed that 4% were attacked by *Ps. caryophylli*, 48% were perfectly healthy, while in 48% one or a few brownish vessels were noticed in each cutting; these could not with certainty be said to be infected by bacteria. A simultaneous biological examination of the same

cuttings disclosed, however, that 48% (against 4%) of the cuttings were attacked by *Ps. caryophylli*, while only 24% (against 48%) were healthy, and 28% (against 48%) with brownish vessels gave a positive bacterial reaction, though *Ps. caryophylli* could not be demonstrated. Thus, the number of perfectly healthy cuttings was halved (from 48 to 24%), at the same time it appeared that there were not only 4% but even 48% of the plants from which it was possible to isolate *Ps. caryophylli*.

As many of the dubious cases, i. e. the cross sections with few faintly brownish-yellow vessels, and perhaps also some of the cuttings which, when examined under the miscroscope, appeared to be healthy, must nevertheless contain *Ps. caryophylli*, it might be interesting to see on what points the microscopic analysis failed. An analysis was therefore made, the so-called "method test" of 4 ordinary lots of cuttings of the variety "Harvest Moon", 25 cuttings being chosen at random from each lot. Each of these 100 cuttings was then examined according to 4 different methods: by microscopy of cross sections of stem (the so-called "microscopy method", (= "M")) and by putting cross sections of stem[1]) partly onto PDA in test tubes (the "agar method" (= "Agar")), partly into nutrient broth in test tubes (the "broth method" (= "Broth")), and finally, a check (the "suspension method" (= "S")) was made by putting cross sections of stem into sterile, distilled water, which was then streaked on to PDA. The results of the different examinations were characterized in four different ways: "+" indicating that *Ps. caryophylli* was found with certainty, and "(+)" indicating that bacteria were found in connection with the cross sections of stem cut off, but that the presence of *Ps. caryophylli* among them could not be established with certainty. The plants which in the microscopy appeared to have few brownish-yellow vessels were denoted by "?"; "—" indicates nothing abnormal could be found during microscopy or that the discs on agar or in nutrient broth or water remained sterile. The results of the entire investigation (also called the "method test") are listed in Tables IX and X, the latter containing a more summary review of the distribution of diseased and healthy cuttings, according to the method on which they were examined.

It is evident from Table X that, compared with the biological methods of examination, the "microscopy method" constantly "indicates" too few infected plants and too many "healthy" plants and "cases of doubt". A further study of Table IX also shows that the cross sections which

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[1]) Of course, the stems in question had first been carefully sterilized on the surface by being rinsed with 70% ethanol + 1‰ 8-hydroxy-quinoline sulphate.

Table X. The "Method Test".

A survey of test results for 4 lots (I, II, III, & IV) of 25 cuttings of the variety "Harvest Moon', tested according to 4 different methods: The "microscopy method" ("M"), the "agar method" ("Agar"), the "broth method" ("Broth"), and the check, the "suspension method" by streaking an aqueous suspension on to PDA ("S").

Lot No.	Testing results	No. of cuttings			
		"M"	"Agar"	"Broth"	"S"
I	+	12	14	19	19
	? & (+)	6	7	5	5
	—	7	4	1	1
II	+	13	15	17	16
	? & (+)	4	6	6	7
	—	8	4	2	2
III	+	4	4	12	12
	? & (+)	11	11	9	9
	—	10	10	4	4
IV	+	0	3	4	4
	? & (+)	12	16	17	17
	—	13	6	4	4

\+ *Pseudomonas caryophylli* demonstrated.
(+) bacterial growth (species uncertain) demonstrated.
? brown vessels.
— healthy cross sections of stem, or no bacterial growth on medium.

in the microscopy examination are characterized by "?" were found partly to be sterile (see IV-18) and partly to be attacked by bacterial stem-crack (see I-11); finally, there were some cross sections which gave a positive bacterial reaction while the presence of *Ps. caryophylli* could not be demonstrated (see I-8). Actually, these facts presented no obstacle to the application of the "microscopy method" as a reliable check on the occurrence of disease in carnation cuttings. In the routine examinations all the cases of doubt might then be included among the easily noticeable cases of disease, so that only the cuttings whose cross sections were found to be absolutely normal were "approved" and propagated in the usual manner. Such a procedure would be acceptable if a healthy appearance of the cross sections indicated that the cuttings in question were really healthy. It appeared, however, (see Table IX, e. g. I-6, I-19, III-7, IV-10, etc.) that some of the cross sections which when examined under the microscope must be designated as perfectly healthy,

actually contained bacteria, which were isolated by means of the biological methods and determined to be *Pseudomonas caryophylli.* This provided the final proof that the microscopical examination was so unreliable that it was not directly applicable in practice. It is true that, by the examination under the microscope, the greatest number of diseased cuttings could be sorted out from a lot, but the small number of cuttings which were too faintly infected to be found by microscopy presented so great a risk of dissemination of the infection during the rooting period on the propagation bench that the method cannot be recommended in general. In small nurseries, which cannot afford the more complicated biological check, the "microscopy method" may nevertheless be used, but it is then a prerequisite for obtaining a successful result that the selected, supposedly healthy cuttings are rooted each in a pot of its own, which should be placed on a particularly well drained bench. Furthermore, the plants will have to be kept under observation for 2 or 3 years, during which the infected plants will generally show visible signs of infection, so that they may be discarded. From the remaining, highly selected, plants it will be advisable only to take top cuttings, which should be subjected to another microscopical examination; in certain cases it will even be necessary to apply the whole isolation technique described above to several generations of cuttings. Provided the benches, flower-pots and soil used are thoroughly sterilized, it will be possible, perhaps in the course of 3 or 5 years to eliminate bacterial stem-crack from a certain carnation variety by means of the "microscopy method".

The "microscopy method" is thus far too unreliable, slow and space-consuming and, consequently, too costly for large, modern carnation nurseries which change the varieties so quickly that the long examination time required by the "microscopy method" (at least 2 or 3 years) can hardly be tolerated. Besides, they produce very large quantities of cuttings by placing them close together in a common medium on large propagation benches, a method which offers great advantages from a cultivation-point-of-view but also involves a considerable risk of a very effective dissemination of both bacterial and fungous diseases. It would therefore be highly desirable if an examination method could be arrived at, which was cheaper and quicker and which, above all, was able to point out reliably the diseased carnation cuttings among the healthy ones, so that there was no longer any reason to be afraid of propagating the selected, healthy cuttings in the usual way.

c. The "Agar Method"

It has been mentioned already that, besides by optical examination of cross sections of stem, the interior sterility of carnation cuttings can also be investigated by biological methods. As the former method proved not to be sufficiently reliable, it was natural to try whether it would be possible to isolate the pathogen sufficiently reliably by the traditional isolation technique, and whether such a comparatively complicated investigation technique was applicable in practice to large quantities of cuttings. It should be added in this connection that the isolation technique used so far by the author for *Ps. caryophylli* consisted in the cutting-off of small discs of stem from carnation cuttings rinsed with ethanol (70% ethanol + 1‰ 8-hydroxy-quinoline sulphate). The discs were then partly placed directly on PDA in Petri dishes, partly transferred to a few millilitres of sterile, distilled water in test tubes. After about 6 hours at room temperature, a few drops of the aqueous suspensions were streaked on to PDA in Petri dishes. In December 1953 these two isolation methods were used for a comparison with the optical examination method. Simultaneously, the description recently given by the American E. F. GUBA (1952) of an investigation method was studied. Like one of the author's methods, the American one was based on the placing of discs of stem on PDA. GUBA's method, which was stated to be especially suitable for mass-examination of carnation cuttings, is carried out so that, first, the surface of the lowermost 5 cm of the stem is sterilized by being dipped into a 1‰ solution of mercuric chloride for 30–60 seconds; next, the lowermost 10 to 15 mm of the stem is cut off with a flamed scalpel and discarded. With another flamed scalpel, 2–3 discs, about 2–3 mm long, are then cut off and placed on sloped PDA in test tubes by means of a flamed needle. While the investigation is going on, the cuttings are placed in a refrigerator at a temperature of about 1–2°C, but GUBA's fails to indicate how the cuttings are placed in the refrigerator—whether they are stored under dry or moist conditions, how the numerical order is maintained, etc. After 7 days, all the discs of stem are examined, and in cases where growth of bacteria or fungi is found round the discs, the cutting concerned is discarded. The other cuttings, whose discs of stem remain sterile, are considered healthy and may therefore be rooted in the usual manner.

GUBA's method deviates somewhat from that of the author, and it was therefore deemed proper to investigate, simultaneously with the current experiments, whether GUBA's method of cutting off the cross

sections of stem was more suitable for mass-examinations than cutting them off with a pair of scissors, as the author had done hitherto. It was also investigated whether test tubes with sloped agar were preferable to Petri dishes. The difference between the sterilization technique of GUBA and that of the author is mentioned on page 89. The comparative experiments showed clearly that in the majority of cases it was possible, by the placing of cross sections of stem (the "discs") on PDA, to find a larger number of infected plants in a certain lot of carnation cuttings than by examination of a cross section of stem under the microscope (cf. Table X). Furthermore, it was noticed that the placing of the discs of stem was far more easy in Petri dishes than in test tubes, in which it was moreover often difficult to make the discs remain on the sloping agar surface; the discs simply slid down to the bottom of the tubes as soon as these were touched. Sloped agar is thus unsuitable for mass-examination by the "agar method". Finally, it may be mentioned that the author's method of cutting off the discs with a flamed pair of scissors direct into test tubes (Fig. 14) or Petri dishes was a far quicker and better method than the cutting-off with scalpels or razor blades, because, with the latter method of cutting, the discs were often inclined to land outside the Petri dishes or test tubes. Whether GUBA's method or that of the author was used, a good deal of time had to be spent in any case in placing the stem-pieces with a flamed needle on the agar and pressing them firmly into position.

Also in these investigations PDA proved to be an excellent medium for *Ps. caryophylli*. The bacteria grow excellently on this medium and form relatively quickly the very characteristic dark-brown colonies, whereby it is possible to see immediately whether it is *Ps. caryophylli* or another species which is present. During the routine examinations it was, however, observed that *Ps. caryophylli* colonies were frequently overgrown by or intermingled with other species of bacteria or "contaminations" so that their brown colour could not be seen distinctly, whereby direct determination was rendered impossible.

By streaking on to agar plates (PDA) of the bacteria from these "mixed colonies", which also occurred in great numbers in the investigation of the diagnostical reliability of the various testing methods (the "method test") it was ascertained that the "mixed colonies", in Table IX denoted by "(–)", almost exclusively consisted of saprophytic ("foreign") bacteria, but also that in a few cases they contained *Ps. caryophylli* (cf. Table IX: I-25, III-23). A further study of Table IX shows that the "agar method" and the "microscopy method" in several instances (cf. Table IX: I-10, I-19, II-13, III-20, & III-21) "overlooked" the regular

Fig. 14. Cutting-off of stem-pieces directly into a test tube with nutrient broth. The photograph was taken during the first testing carried out commercially in a Danish nursery.

cases of disease, which could mostly be ascertained only after the discs of stem had been immersed in water and the bacterial suspension thus produced had been streaked on to PDA.

The author has tried to find out why now and then bacterial colonies failed to appear round certain discs of stem on PDA, though from several discs, from the same cutting, colonies of *Ps. caryophylli* occurred after the discs had been immersed in water and the suspension had been streaked on to PDA; indeed, in certain cases it was even possible to see directly through the microscope that a few vessels in the said discs of stem were attacked by bacteriosis (cf. Table IX: III-6 and III-20). Each individual case of failure of the "agar method" was therefore investigated thoroughly, and a few of the negative results were found simply to be due to the fact that the cut surfaces of the said discs of stem were so unevenly cut off and that, besides, the discs were so insufficiently depressed into the agar medium that the infected vessels did not touch the surface of the medium at all. In other cases, the explanation was undoubtedly to be found in the fact that the discs of stem contained so few bacteria that their growth was not sufficient to manifest itself visibly on the agar surface during the 7 days that passed before the test was terminated.

The conclusion of the investigations mentioned in the above must therefore be, firstly, that the method of investigation suggested by GUBA is less convenient and less suitable for mass-examination than the author's "agar method". Secondly, it is possible, by means of both "agar methods" to find a greater number of cases of disease than by means of the "microscopy method". In addition, the "agar method" is generally also more

reliable than the "microscopy method", amongst other things because it is often possible by means of the "agar method" to see directly whether it is the "proper" species of bacterium one is faced with. On the other hand, it is not possible by means of either "agar method" to point out so many diseased individuals from among a certain lot of cuttings (cf. Table IX I-10, I-19, II-13, III-20, & III-21) as can be done by immersing the discs of stem in water or nutrient broth and then streaking these suspensions on to PDA. Adding to this that preparation, draining-off and sterilization of the agar medium – let alone the cleaning of the glassware that had contained an agar medium – are both expensive, slow and cumbersome, it must be admitted that the "agar method" is not immediately attractive to the carnation grower.

The author is aware, however, that also in the U.S.A. tests are being made with various agar methods resembling those of the author a good deal, but nothing certain is available as yet about the results obtained. On the other hand, if the "agar method" is combined with a strictly separate propagation of each individual cutting which, besides, is kept under observation for a long time, it is, undoubtedly, possible also by means of this method to eliminate bacterial stem-crack from smaller lots of carnations.

In this connection it should be added that, although both the "microscopy method" and the "agar method" are able, separately, to indicate the greater majority by far of diseased individuals from a lot of cuttings, carnations are, as mentioned already, nowadays propagated in a way which is favourable to the spreading of the disease, and if only a few infected cuttings pass the examination and are placed on the propagation bed, there is therefore a great risk of extensive dissemination. The effectiveness of these two control methods is therefore, in spite of everything, not sufficient to ensure the rapid eradication of the disease which is so important to a modern carnation nursery. It is of little avail for the grower to spend time, space and money on having a certain carnation variety freed of the disease, if the cultivation value of this variety has become materially diminished because new and improved varieties have conquered the market during the long waiting time.

d. The "Suspension Method"

As mentioned above, the aim of the control of the bacterial stem-crack of carnation is not only to get rid of the disease in some way or other, but also to do so quickly, that is to say, in the course of one or two

generations of cuttings. During his experiments carried out so far, the author has therefore in his efforts to arrive at a practical control method constantly endeavoured not only to find an inexpensive method which could be applied directly in the nursery, but also a method which was so reliable and rapid that after the first or second testing of the cuttings, the disease would be entirely eliminated. It seems possible to attain this ideal in a rather high degree by examining the cuttings in the way that 2 or 3 small pieces are cut off from each stem directly into sterile, distilled water in test tubes, after which the water (the suspension) is streaked on to PDA in Petri dishes (the "suspension method"). In the experiments described above (the "method test", Tables IX & X: "S"), this isolation method served as sort of a check on the other methods, and by means of it, it was always possible to point out a larger number of infected cuttings from the lot of plants concerned than was possible by either of the other two methods.

There can therefore be no doubt that, in respect of reliability, the "suspension method" is preferable to the two other methods, but, in addition, it presents several practical advantages which in a high degree makes its application in ordinary nurseries possible. In the first place, the discs of stem are easily and quickly cut off with a pair of scissors directly into test tubes, thus avoiding the individual positioning by means of a needle. Secondly, test tubes are much easier to handle and keep in numerical order (in racks) than are Petri dishes, and test tubes are also less expensive than Petri dishes. Finally, it is extremely easy to fill liquid into test tubes and sterilize them all at one time. The most difficult part of the "suspension method" is the streaking-on of the aqueous suspension to the agar medium in Petri dishes. It is no easy matter for nurserymen to prepare the medium and fill it into Petri dishes and, generally, avoid confusion of the many hundred dishes which would be involved. Finally, it should be added that the aqueous suspensions should preferably be allowed to stand for 6–8 hours before they can be streaked on to the agar, which will also be somewhat inconvenient in practice.

The author therefore tried to find out whether it would be possible to avoid the inconvenient streaking-on by replacing the water in the test tubes with a liquid nutrient medium, so that "extraction" and cultivation of bacteria from the small discs of stem might take place only in this medium. If such a method could be adopted, direct inspection of the tubes should be sufficient to judge whether or not the cuttings in question were infected with bacteria.

e. The "Broth Method"

In the endeavours to find a suitable nutrient medium in which to put the discs of stem, an experimental series was included in the "method test". In this experimental series the discs of stem from cuttings were cut off directly into test tubes (15 × 155 mm), each containing about 5 ml nutrient broth (meatextract-peptone-water) (Difco), a medium in which *Ps. caryophylli* will grow exceptionally quickly and which is so fluid that it might replace water, in respect of ability to penetrate into the vessels of the stem-pieces. As a check on this method, the so-called "broth method", streaking-on of all the media to PDA was done for safety's sake, and the results of these streakings are listed in Tables IX & X in the columns marked "Broth". It will be seen that when the "broth method" was used, a greater number of infected cuttings were actually found than was possible by putting the discs of stem in water and streaking the suspension ("S") on to PDA. Otherwise, there was very good agreement between the results of the two methods—or their ability to indicate diseased cuttings from the healthy ones. It further appears from Table IX that when the "broth method" was used it was also necessary to record bacterial growth "(+)" in a great number of broth tubes from which it had not been possible to isolate *Ps. caryophylli* by streaking-on to PDA. This fact was also apparent in connection with the streaking-on of the aqueous suspensions. As demonstrated later, these bacteria originated from inadequate surface sterilization of the carnation stems, but as it had not been possible to demonstrate the presence of any carnation pathogen in numerous random samples, the species of bacteria were not determined, but were merely recorded as "contaminations".

In the few cases where the discs of stem had caused no growth of bacteria in the nutrient broth, the latter remained perfectly clear (sterile); it was therefore easy to separate these tubes, which represented the completely healthy cuttings, from the tubes in which the medium was turbid with bacteria. The state of health of the cuttings thus separated was, however, checked once more by renewed isolation tests, in which discs of stem were put into water, which was then streaked on to PDA, and by examination of cross sections of stem under the microscope, but among the test plants from the "method test" or among the plants which were sorted out during the first mass-investigations it was impossible to find a single one which was attacked by *Pseudomonas caryophylli*. For the time being, it is therefore possible to establish that although the "broth method" must be said to be so sensitive that besides the actual "cases of disease", it will also record a great deal of "contamina-

tions", it seems, on the other hand, to offer some guarantee that all infected cuttings are found and, consequently, to provide the possibility to free a certain lot of cuttings from the infected ones in the course of one or two generations.

When the method is used in practice, it should be observed that it is impossible directly to distinguish between the tubes which represent the actual cases of disease and those representing the contaminations, because all the media will display almost uniform colour and turbidity. In practice, it will therefore be necessary to discard all the cuttings whose discs of stem have somehow produced bacterial growth in the medium. The appearance of the medium in the test tubes—clear or turbid—thus provides the possibility of a quick and distinct assessment of the cuttings; on the other hand, some of the disease-free cuttings are discarded in this way.

Investigations were therefore made to find out whether contaminations could be reduced somewhat by intensifying the surface sterilization of the cuttings, and whether these could stand such a treatment. A few preliminary tests proved that the cuttings would stand even a very thorough rinsing of their stems with a mixture of 70 % ethanol and 1 ‰ 8-hydroxy-quinoline sulphate, and that in several instances this treatment reduced the number of contaminations to 5–10 %, which at that time was not found to be an unreasonably high figure in proportion to the large number of actual "cases of disease".

The need for a general examination of carnation cultures attacked by bacterial stem-crack had gradually become very urgent, because in certain varieties (e. g. "Harvest Moon") the number of diseased plants among the newly rooted cuttings now generally amounted to between 40 and 60 %. Consequently, although the "broth method" was not yet finally established, it was found desirable to have the carnations examined by means of the outlined "broth method", which, in any case, might be able to reduce the percentage of diseased plants very substantially, if not to eradicate all the diseased plants.

The continued investigations of the practical application of the "broth method" were therefore moved to a nursery, where the testing was commenced immediately at a bench in a greenhouse according to the following investigation procedure:— Immediately after the breaking-off the lowermost 2–3 cm of the cutting were wiped and rinsed very thoroughly with a rag soaked in a disinfectant consisting of 70 % ethanol and 1 ‰ 8-hydroxy-quinoline sulphate, a mixture which, for reasons of convenience, was since called "oxyspirit". The rinsed portion of the

stem was then flamed in a spirit flame, and with a flamed pair of scissors the lowermost 0.5–1.0 cm of the stem was cut off and discarded, in order not to transfer to the broth[1]) any disinfectant that might have been absorbed. After that, 2 or 3 small pieces of about 3–5 mm length were cut off with scissors directly into a test tube with 5 ml nutrient broth plus 1⁰/₀ glucose[2]) (Fig. 14). The test tube was then numbered, placed in a rack (for 50 tubes) and, finally, put in an incubator at a temperature of 30–32°C. In the meantime, the cut-surface of the cutting was then cut clean with a sterile razor blade, and the cutting was placed in water in a 10 ml test tube, which was given the same number as the corresponding test tube with the stem-pieces in the nutrient broth (Fig. 15), and placed in a cool greenhouse.

After 4 days, the "broth tubes" were removed from the incubator and transilluminated, and all the tubes with turbid media were taken out. All cuttings bearing the same numbers as the turbid media were then removed from the racks and burnt. The rest of the cuttings, which thus corresponded to the sterile media, were assumed to be healthy and were placed in the usual manner on sand beds made of entirely new materials.

In this way, the first 500 cuttings were tested; random samples from the turbid media showed, however, that in practice the contamination percentage could not be kept as low as desirable. Frequently, a contamination percentage of even 10–40$^0/_0$ was ascertained, and it was then tried whether a combination of the sterilization method of GUBA and that of the author was able to reduce these rather high figures and the consequent waste of cuttings. The sterilization process was then extended so that the lowermost 3–4 cm of the stem were first dipped in 100 ml 1$^0/_{00}$ aqueous solution of mercuric chloride for 20–30 seconds in a 150 ml beaker, after which the mercuric chloride was washed off in another beaker containing "oxyspirit". After this the already described sterilization, consisting of wiping of the stem with "oxyspirit" and flaming of the stem, was made (Figs. 14 & 15). This extended sterilization method did not delay the work very much, nor did the cuttings suffer thereby. Random samples showed, however, that the percentage of contaminations was now reduced to 10–20$^0/_0$, a waste which, of course,

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[1]) In his paper, GUBA (1952) has proved, as confirmed by the present author, that mercuric chloride is not taken more than 1–3 mm up into the stem during a treatment period of 3–4 minutes.

[2]) The glucose was added because in the interim it was found out that 1–2$^0/_0$ glucose accelerated the growth of the bacteria considerably.

Fig. 15. Apparatus for "nutrient broth testing". From left: Cuttings washed with "oxyspirit" ($70^{0}/_{0}$ ethanol + $5^{0}/_{00}$ (previously $1^{0}/_{00}$) 8-hydroxy-quinoline sulphate); beaker with $5^{0}/_{00}$ (previously $1^{0}/_{00}$) mercuric chloride solution; rack with 50 short test tubes containing boiled water for cuttings; beaker with "oxyspirit"; glass dish with "oxyspirit" and cotton wool; spirit lamp; glass dish with "oxyspirit"; two pairs of scissors, and rack with 50 ordinary test tubes with 5 ml nutrient broth per tube.

it would be desirable to have reduced further, but which, in the circumstances, could hardly be lowered much more. On the other hand, these contaminations involved but a slight increase in the cost of the "approved" cuttings.

In the spring of 1954 the "broth method" was adopted for the testing of a very large number of cuttings, altogether 15,207, distributed on 9 different varieties (Table XI). Out of this number, $29.3^{0}/_{0}$, on an average, was discarded, including actual bacteriosis and contaminations. The total result of the testing is shown in Table XI, from which it appears that the percentage of discarded cuttings (per rack of 50 tubes) has varied a good deal, both within the same variety and within all the varieties, the lowest figures being usually $0–4^{0}/_{0}$ (in a few cases even as much as $36^{0}/_{0}$), while the highest percentages varied between $12^{0}/_{0}$ and $100^{0}/_{0}$.

While the above-mentioned investigations were proceeding, random samples were regularly taken from the turbid media and subjected to a check examination, whereby it was once more ascertained that the contaminations usually increased the number of discarded cuttings by $10–20^{0}/_{0}$. Furthermore, regular check testing was also made of the "approved" or healthy cuttings, but it was never possible to demonstrate attacks of *Pseudomonas caryophylli* among these cuttings. The author

Table XI. Review of testings made according to the "broth method" in the period January 14, 1954–March 17, 1954.

(Test tubes and cuttings are standing in racks, each holding 50 tubes, and the percentage of discards within each lot of 50 cuttings is stated separately).

Variety of carnation		Percentage of cuttings discarded per rack of 50 cuttings		
Name	Total No.	Minimum %	Maximum %	Average %
Yellow Sim	1,000	36	86	54.9
Peppermint Sim	750	2	50	26.0
Petersen's Pink Sim	1,350	2	28	13.5
Dark Red Sim	4,085	4	70	18.9
White Sim	1,410	2	75	22.0
Cardinal Sim	450	36	100	70.4
Geiger Sim	838	4	43	28.0
Harvest Moon	4,094	2	66	25.8
Sidney Littlefield	1,230	0	12	4.6
9 varieties in all	15,207	0–36	12–100	29.3

has, however, not been able to follow the development of all the 10,000 cuttings which during the testing were declared to be healthy, but about 3,000 of these plants of the variety "Harvest Moon" were planted out in a nursery near Copenhagen, and could thus be regularly inspected. These plants have now, in December 1956, been standing for nearly 3 years without it having been possible for the author to demonstrate bacteriosis in a single plant. With regard to the other cuttings, which were examined in the spring of 1954, no information is available about withered plants, but about a lot of 15,000 cuttings which were tested in 1955 by the same method as used in 1954, and which were all sent to a nursery in England, the author has been informed verbally that a single plant has died in consequence of attack of *Ps. caryophylli.*

Accordingly, it cannot be taken for granted that it is possible by means of the "broth method", and by the first testing, to indicate with absolute certainty all the diseased plants in a lot of cuttings, but obviously the safety factor is considerably higher than for the "agar methods".

The "broth method" was, however, far from being finally worked up. During the work of examining the large number of cuttings in the season 1953–54 (15,000) and in the season 1954–55 (40,000 in one nursery and

100,000 in another), it happened now and then that on certain days the number of contaminations suddenly rose to both 80% and 100%, although other cuttings of the same lot and under exactly the same investigation conditions displayed quite normal and relatively low contamination percentages (10–20%). Each time attempts were made to find the cause of the abnormally high number of contaminations, and it then appeared time and again that the great number of contaminations were due to the fact that before the testing the cuttings had somehow been in contact with water, some of which had been absorbed. As ordinary water is never sterile, the biological investigation of the interior sterility of the stems will record the content of bacteria from the water, whether the "agar method" or the "broth method" is used and whether the cutting is completely healthy or not. When employing biological investigation of the sterility of the tissues of carnation stems it is therefore a first requisite that the cuttings are kept completely dry from the breaking-off from the mother plant to the time when the testing is done. This involves, for instance, that the cuttings must be tested on the very day they are broken off, and that they are at no time before the testing placed in buckets with water or in boxes with moist paper—which is a very common practice to avoid desiccation of the young shoots. Nor can cuttings in plastic bags for cold-storage be used in the biological test, seeing that the vapour-saturated air in the bags is sufficient to contaminate the vessels of the cuttings. Neither is it possible to test the tops of newly rooted cuttings, because the vessels of the young plant still contain a large number of bacteria (harmless) taken up with the water from the propagation bench.

Now, the assumptions for the application of the "broth method" and the detailed investigation process were eventually tried out to such an extent, and its reliability so well-founded, that other carnation growers began to take an interest in its adoption, and in February 1955 a newly-built laboratory for the testing of carnations according to the "broth method" was inaugurated in another nursery. This laboratory was equipped with autoclave, incubator (Fig. 16), sink and drying-cupboard for glassware, etc. At the same time, a single link in the chain of the "broth method" was improved. The ordinary cotton-wool plugs hitherto used for closing the test tubes with media were replaced with aluminium caps (16 $\times$ 30 mm) (Fig. 16), which, on account of their great durability, are much cheaper than cotton-wool in the long run and, besides, are far quicker, and consequently also cheaper, to use during the preparation of the test tubes with media.

Fig. 16. Incubator room in a Danish carnation nursery. Note the broth tubes in racks of 50, and that the tubes are closed with aluminium caps.

As the testing in the new laboratory was carried out under the best possible conditions (Fig. 17), the number of the very annoying contaminations was expected to be reduced somewhat; and this was really the case in the first couple of months, but then again it began rising evenly and fast until, in May 1955, it reached between 70% and 100%! Renewed investigations to find the cause of the violent and unexpected rise were particularly directed against the ability of the aluminium caps to seal the test tubes properly. 200 cuttings were tested with one-half of the test tubes closed with aluminium caps and the other half with the ordinary cotton wool plugs, but it proved impossible to find any difference between these two types of closure, both sets having a contamination percentage of 80–82%. Further investigations of the number of germs in the atmosphere of the laboratory and the incubator, of the efficiency of the autoclaving, as well as of the sterility of the media and the disinfection agents disclosed nothing abnormal. The question then remained whether the surfaces of the cuttings carried some exceptionally resistant bacteria which were either well hidden or which the sterilization methods used so far were unable to kill. To have these points cleared up, the wax layer from the lowermost internode of 50 carnation stems was completely peeled off, after which the wax layer from each cutting was streaked on partly to PDA and partly to nutrient agar in Petri dishes.

Fig. 17. Testing by the "broth method" in a Danish nursery. The lady in the background is rinsing the cuttings and cutting off stem-pieces. The lady in the foreground is "trimming" the cuttings with a sterile razor blade. The cut-surface of the cutting is thus cut clean and the lowermost leaves removed, so that the cutting is fit for setting in the sand.

The few and very variegated bacterial colonies that appeared thereby seemed in no way to warrant the very high number of contaminations ascertained in the routine tests, and which were apparently produced solely by a few species of *Bacillus* (*B. subtilis*, *B. megatherium*), and which appeared only in very limited numbers in the Petri dishes. The search for the cause of the appearance of the strange contaminations was then directed against the anatomic structure of the carnation stems, and here, eventually, the bacteria were found to have an excellent hiding place in the deep pit-shaped cavities above each stoma in the epidermis of the stems. These pits were almost a dozen microns deep and nearly as wide and were found in a number of no less than 20–26 (sometimes even 38–40) per square millimetre surface of stem (Fig. 18, p. 91).

Hence, it was obvious that, on account of its greater surface tension, the aqueous solution of mercuric chloride had not been able to penetrate into the hollows in epidermis and kill the bacteria there. Nor had the very brief treatment with "oxyspirit" been able to sterilize these cavities sufficiently well. GUBA's sterilization technique, with a single washing with mercuric chloride alone must therefore be considered quite inadequate. Thus, the cause of the greatly fluctuating sterilization results was to be found in the insufficient penetration of the liquids used into the epidermis cavities, and some test were made to verify this hypothesis.

The test comprised altogether 400 cuttings of the variety "Salmon Sim", which in the check isolations made during the routine testing had proved to be completely free from disease while 80–100$^{0}/_{0}$ of the media had become turbid with bacterial growth. Out of these cuttings, 100 were treated by the "broth method" as usual, another 100 were treated in the same way after being thoroughly washed and wiped with 1$^{0}/_{00}$ "oxy-spirit" before treatment with the mercuric chloride. Before being subjected to the usual "broth method" test the remaining 200 cuttings were wiped with 1$^{0}/_{00}$ "Rodalon" (benzalconium chloride, a quaternary ammonium compound). These washings served partly as an additional sterilization and partly as a reducer of the surface tension, so that the solution of mercuric chloride might penetrate into the cavities of the epidermis. The results of these additional washings were amazingly good. The number of tubes with bacterial growth in the medium were reduced thereby to between 0 and 6$^{0}/_{0}$ (in a single instance, however, 16$^{0}/_{0}$ were found) for the two lots of cuttings which had been washed specially, while the lot which had been treated according to the methods hitherto used continued to display a contamination percentage of 80–82$^{0}/_{0}$. There was but slight difference between the Rodalon-treatment (0–16$^{0}/_{0}$) and the washing with "oxyspirit" (4–6$^{0}/_{0}$), but as "oxyspirit" had been used already for the other washings, and as in the subsequent mass-examinations the contamination percentages with the spirit-washing were constantly a little lower than for the cuttings washed with Rodalon, it was decided to augment the sterilization process of the "broth method" by a preceding washing of the cuttings with "oxyspirit". It may be added that for the last 37,000 cuttings tested (spring 1956), the contamination percentage has mostly varied between 0 and 8$^{0}/_{0}$ (in quite a few cases up to 16–24$^{0}/_{0}$), so the average has been about 1.6$^{0}/_{0}$.

However, later on it was noticed that especially in summer time it was difficult constantly to check the contaminations in such a degree, but when the concentration of both "oxyspirit" and mercuric chloride was increased to 5$^{0}/_{00}$ (instead of 1$^{0}/_{00}$) it has been possible, in general, to keep the amount of contaminations below 2 per cent.

In its final form, the "broth method" is then as follows:— The lowermost internodes, or not more than 3–4 cm, of the stems of 50 newly broken off, dry cuttings are first washed thoroughly with an autoclaved rag soaked in "oxyspirit" (= 70$^{0}/_{0}$ ethanol + 5$^{0}/_{00}$ 8-hydroxy-quinoline sulphate). Next, one cutting at a time is placed in a 150 ml beaker with about 100 ml 5$^{0}/_{00}$ aqueous solution of mercuric chloride and after

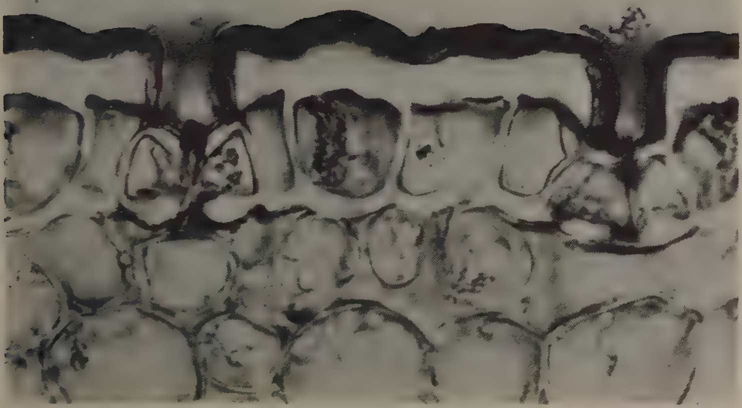

Fig. 18. Cross section of stem of "Petersen's Pink Sim" showing how deeply the stomata are sunk in the epidermis of a carnation stem. Cuticle about 4.5 μ thick and stained with Sudan III. $\times 1000$.

20–30 seconds (never more than 45 seconds and the liquid should not touch that part of the stem, which is not wanted to be sterilized, because the cutting may then be harmed), the cutting is taken out and rinsed in another 150 ml beaker filled with "oxyspirit". Then the lowermost internode is wiped very thoroughly with a small rag soaked in "oxyspirit"; this liquid is finally burned off over a spirit flame[1]). At the same time, a pair of nail-scissors (of stainless steel[2]), with slightly curved jaws) is flamed and used for cutting off 1 cm of the stem (cf. upper foot-note on p. 84). This small stem-piece is discarded, after which 3 more stem-pieces, each 5–10 mm long, are cut off into a test tube (15 × 155 mm) with about 5 ml nutrient broth + $1^0/_0$ glucose, the test tube is then closed at top with an aluminium cap (16 × 30 mm). After the cutting-off of the stem-pieces, the scissors are placed in a dish with "oxyspirit" until the next flaming and clipping are to take place. Then the test tube

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[1]) The limitation of the sterilization to the lowermost internode or to the lowermost 3–4 cm of the stem presents the advantage that the distal part of the cutting, which is later on to be rooted, is not subjected at all to the somewhat drastic sterilization.

[2]) As 8-hydroxy-quinoline sulphate will corrode ordinary steel but not stainless steel, it is important that the scissors are made of the latter material. Incidentally, it is most convenient to use two nail-scissors.

with the 3 stem-pieces is numbered and placed in a steel-wire rack capable of holding 50 tubes. The cut-surface of the cutting from which the stem-pieces have been clipped off is cut clean with a sterile razor blade—a new one for each cutting—and placed in ordinary, boiled tap-water[1]) in a 10 ml test tube, which is given the same number as the corresponding test tube with the stem-pieces. Also the test tubes with the cuttings are placed in a rack holding 50 tubes; when full, the rack is placed in a light and cool room or greenhouse. The test tubes with nutrient broth (and the stem-pieces) are, however, placed in an incubator at a temperature of 26–27°C, and after 4 days the number of turbid tubes in each rack is roughly estimated. If only 0–2% of the tubes are turbid, the test can be concluded after the 4 days, because numerous check tests have shown that the 0–2% have so far represented contaminations only and not bacteriosis proper. If, however, there are more than 2% turbid tubes (media), the samples should be left in the racks for another 2 days, i. e. 6 days in all, after which all the tubes are taken out and transilluminated against a black blackground and in front of an ordinary electric-light bulb (15–25 decalumen), during which the numbers of the tubes with turbid media are noted. Afterwards, the cuttings whose water-test-tubes bear the same numbers are removed and burnt. Thus, the rest of the cuttings correspond to the sterile "broth tubes" and are declared healthy—"approved"—and can therefore be inserted in sand in the usual way. The "broth method" is best carried out by two persons, one of whom sterilizes and cuts off the stem-pieces, while the other flames the test tube, holds it while the stem-pieces are being çut directly into it, and prepares the cuttings so that they are ready for setting before being put into water in the test tube (Fig. 17). The persons who have so far carried out the investigations have been girls without any special training in bacteriology and laboratory technique, as such knowledge has proved to be unnecessary. Experience shows, furthermore, that it is possible for two assistants to examine 600 nutrient media, sort the relevant cuttings and prepare another lot of 600 cuttings in one workday[2]). Besides this, the same two assistants can

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[1]) The use of boiled water is recommended, as some algae and slime formations on carnation stems have been found after 6 days in certain cases where raw water has been used; it has, however, been possible entirely to avoid this contamination by using boiled water.

[2]) When testing is done also for fungous disease (*Phialophora*), only 400 cuttings can be examined in a day. However, 3 assistants can prepare media and examine 600 cuttings per day.

prepare the quantities of media and liquids required, as well as clean the tubes.

It will appear from the description above that the "broth method" is only able to indicate whether a lot of carnations is attacked by bacteria and in what percentage the disease is present, but it is not possible immediately to see to what species of bacterium the disease is due. For this purpose, also the "broth method" has to be supplemented by streaking-on to PDA in Petri dishes and so the method can be used as either a quantitative or qualitative analysis of disease. The streaking analyses may, however, then be made on all samples, and as it is so fortunate that *Pseudomonas caryophylli* forms particularly easily recognisable colonies on PDA, these investigations will not require bacteriologically trained personnel either.

It will further be noticed from the description of the method that the temperature in the incubator has been determined to be 26–27°C, although the optimum temperature of *Ps. caryophylli* lies between 30 and 37°C, but, with a view to the simultaneous application of the method to the other vascular bacteriosis of carnations (the *Pectobacterium* species (cf. Chapt. II)), it has proved necessary to incubate at a somewhat lower temperature, because 26–27° is the optimum temperature for the growth of this latter bacterium, which is inhibited by higher temperatures. In return, it is necessary to allow *Ps. caryophylli* a somewhat longer time to grow sufficiently, so that even in stem-pieces with the slightest degree of infection the bacterial growth becomes visible in the medium. Tests with numerous nutrient broth samples have proved that after altogether 6 days, bacterial growth very seldom appears in the tubes (media); in the few cases in which this has happened so far, such bacterial growth has not been caused by *Ps. caryophylli.*

Furthermore it should be observed that only 50 cuttings should be treated at a time, because liquids and rags ought to be renewed after this number has been treated; the rags may be autoclaved and used several times.

The abovementioned testing method—in Danish: "KPV-Metoden",—has now been in use in two Danish carnation nurseries for nearly three years and about 400,000 cuttings have been tested, but until now none of the tested plants grown in Denmark have been found infected with bacterial stem-crack. However, if—as was the case in England—a few of the tested plants nevertheless should appear to carry the disease, the cuttings from the tested mother plants will have to be tested once or twice again, and rooted in small pots (one cutting in each pot). To

prevent the spread of the bacteria, the pots can be placed either hanging in the loops of chicken wire netting or placed on the netting supported by a thick layer of pebbles or pea shingle (Fig. 28, p. 139). These methods of propagation will require much watering or application of the so-called "misting equipment".

If, however, this way of propagation is repeated with two or three consecutive generations of carnations, it should be possible to eradicate the disease completely within 6–18 months.

Chapter II

Bacterial Stunt of Carnation

(*Pectobacterium parthenii* var. *dianthicola* var. n.)

1. Introduction

During one of the author's frequent visits to the Copenhagen nursery where bacterial stem-crack of carnation was discovered for the first time, his attention was caught one day in December 1954 by a lot of some 10,000 carnation cuttings of the variety "Salmon Sim" bought from another Danish nursery as rootless and not especially checked cuttings. At that time these cuttings had been standing on the propagation bench for a couple of weeks, and some of the plants had begun forming roots, but about 60–80% of the plants were dead or dying.

A cursory inspection through a hand lens of cross sections of stems from wilting plants revealed plainly discoloured vascular tissues, and by subsequent microscopical examination of the diseased stems a mass of bacteria was seen in the discoloured vessels. There was much to indicate the outbreak of another attack of bacterial stem-crack of carnation, but a closer examination showed, partly that the vessels were not so badly destroyed as is commonly seen in carnations infected by bacterial stem-crack (cf. Figs. 2 and 19), and partly that it was impossible to isolate *Pseudomonas caryophylli* even in repeated attempts. A different species of bacterium turned up persistently, forming greyish, roundish but distinctly lobate colonies. This type of bacterium appeared immediately, in pure culture, practically speaking, and in the preliminary determination it was referred to the genus *Pectobacterium* (*Erwinia*). Since, however, no carnation disease caused by any species of *Pectobacterium* had been described so far, the isolated bacteria were in the first instance supposed to be harmless saprophytes taken up into the vessels of the cuttings from the water in the propagation bench. But as all the plants were discarded immediately, the true cause of the poor growth of the cuttings was not further investigated.

The not quite typical pathological picture and the fact that *Pseudomonas caryophylli* could not be isolated from the dying plants were recalled to mind when in 1955 the author of this paper was once again faced with a lot of languishing young mother plants of the variety "Lucky Strike" bought as cuttings in Holland but rooted and potted in a Danish nursery.

The infected "Lucky Strike" plants had a decidedly sickly appearance, with narrow, greyish leaves and very short internodes, and gradually many of the plants died. When the stems were cut through, a large number of brownish discoloured vessels full of bacterial slime was found, but the vascular tissue was not decomposed to such an extent (Fig. 19) as in the case of attack of *Ps. caryophylli* (Figs. 2 and 6, pp. 18 and 26). The disease was thus in part reminiscent of the previously described attack in cuttings of "Salmon Sim", and as a species of *Pectobacterium* forming greyish, lobate colonies was again isolated from the discoloured vessels, there were eventually many signs to indicate that the two varieties of carnation were actually attacked by a vascular bacteriosis differing from bacterial stem-crack of carnation. This assumption was borne out when some time afterwards it was observed that some cuttings tested on nutrient broth and originating from the diseased "Lucky Strike" mother plants were likewise found to be dying with vessels full of bacteria, from which the aforementioned species of *Pectobacterium* was again isolated.

This was something quite extraordinary, because it was the first time in Denmark that "nutrient broth tested" cuttings were found not to be perfectly free of bacteriosis, and all the 95 plants, of which the lot with the tested cuttings consisted, were therefore at once taken out of the soil and examined. It was then ascertained that about 13⁰/₀ of the plants had few or many vessels filled with bacteria, and all isolation tests resulted in the appearance of pure cultures of the abovementioned species of *Pectobacterium*.

Hence, there was every probability that the freshly isolated bacterium was the cause of the disease in "Salmon Sim" as well as in "Lucky Strike" and that it was even able to pass the "nutrient broth test" in certain cases! The bacterium was therefore submitted to a closer examination, which proved that the disease in the two varieties of carnation was caused by a species of *Pectobacterium* so far undescribed.

The new carnation pathogen was discovered practically simultaneously in Denmark, Holland and England, and in April 1956 it was described by R. A. Lelliott (England) as a physiological strain of *Erwinia chrysan-*

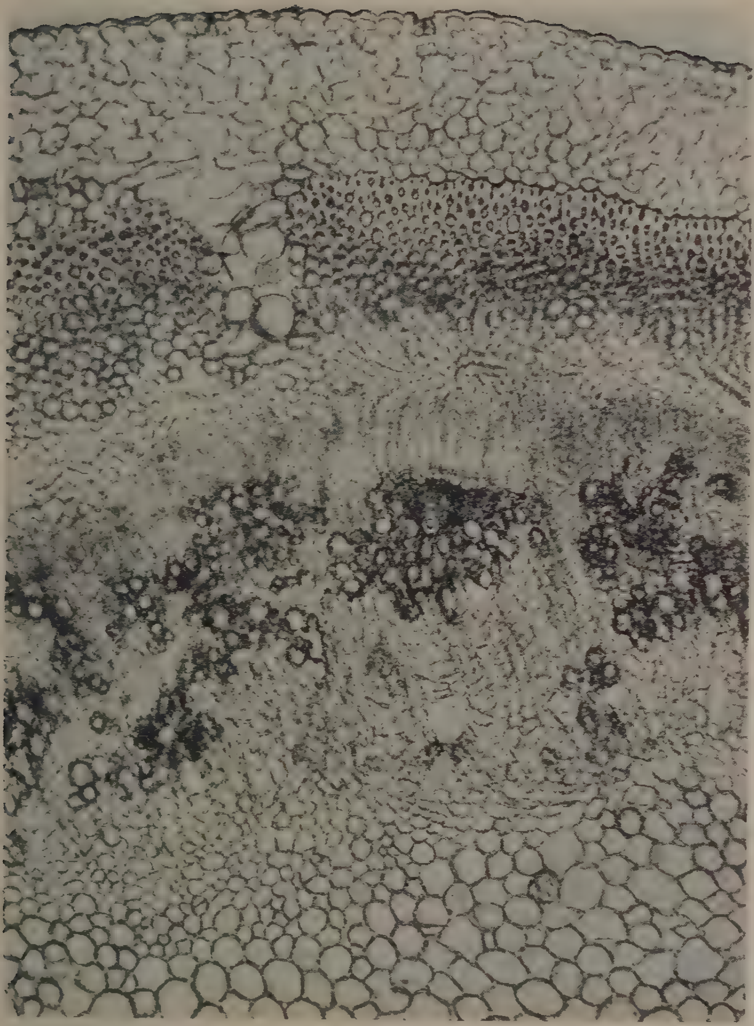

Fig. 19. Cross section of stem of "William Sim" artificially infected with *Pectobacterium parthenii* var. *dianthicola*, isolate No. 98. Note the dark (infected) vessels, surrounded by some meristematic cells. ×150.

themi BURKHOLDER et al. (1953), but no specific name was given to this strain. The author of this paper therefore attempted a closer determination of the bacterium and arrived at the conclusion that the new carnation

pathogen is not identical with any one of the bacteria previously described within the genus *Pectobacterium* WALDEE, and he has proposed (see Sect. 6, p. 129) that this bacterium should be called *Pectobacterium parthenii* var. *dianthicola* var. n.

In the description by LELLIOTT (1956) of the new carnation bacteriosis the disease is termed "slow wilt of carnations" because this bacteriosis often has a longer incubation period than "bacterial wilt of carnation". In this connection it may be observed that in certain cases "slow wilt" develops faster than "bacterial wilt" and that the "wilt disease" produced by various fungi (*Phialophora* and *Fusarium*) will generally also develop extremely slowly and with symptoms that are difficult to distinguish from those of the bacterioses. "Slow wilt" must therefore be said to be a misnomer particularly confusing to nurserymen—for whom common names are coined first and foremost—and the author suggests, in lieu of "slow wilt", to term the disease "bacterial stunt of carnation" (in Danish: "Nellikens Kortskudbakteriose"), the stunted young shoots being the most sure symptom distinguishing this disease from bacterial stem-crack of carnation and from wilting produced by fungi.

2. Description of the Disease

History and distribution. Bacterial stunt of carnation was first observed in Holland in 1951 (BAKKER & SCHOLTEN 1955). The disease was not at once realised to be "bacterial stunt of carnation", the causal bacterium being determined by M. BAKKER and G. SCHOLTEN (1955) as *Pseudomonas caryophylli* (BURKH.) STARR & BURKH., which from America is described as the cause of "bacterial wilt of carnation" or—as the author has now proposed—"bacterial stem-crack of carnation". According to BAKKER & SCHOLTEN's description of the symptoms and of the bacterium there can, however, be no doubt that it is "bacterial stunt of carnation" with which the Dutch plant pathologists have been faced. And after the author personally has had occasion to compare the Danish and the Dutch bacterial isolates and furthermore to discuss these diseases with BAKKER and SCHOLTEN, there cannot be the slightest doubt either, that the Dutch isolates are identical both with the bacterium isolated by the author and that isolated by R. A. LELLIOTT, and which the author has named *Pectobacterium parthenii* var. *dianthicola* var. n. (cf. Section 6, pp. 117–131).

The first time attention was actually drawn to the new vascular bacteriosis of carnations ("bacterial stunt of carnation") was when, in

his lecture on "Bacterial Wilt in Carnations and its Control" given at the 14th International Horticultural Congress at the Hague (The Netherlands) in August-September 1955, the author mentioned the occurrence of a new bacteriosis in Danish perpetual-flowering carnations, found in December 1954 and May 1955 (HELLMERS 1957). Practically simultaneously (in September of 1954 and in the spring of 1955) R. A. LELLIOTT observed this bacteriosis in 11 English nurseries. LELLIOTT was virtually searching for *Pseudomonas caryophylli*, but he often isolated another species of bacterium from the wilting carnations, and in April 1956 he described this new carnation pathogen as a strain of *Erwinia chrysanthemi* BURKHOLDER et al. (1953) and named the disease "slow wilt of carnation".

The occurrence of bacterial stunt of carnation was, as already mentioned, observed in Denmark in December 1954 in the variety "Salmon Sim" and then again in May 1955 in "Lucky Strike", but the disease has undoubtedly been in Denmark several years before. Carnation growers are of the opinion that the disease has erroneously been taken for attacks of *Fusarium* species, but nothing can be said for certain about this assumption because this bacteriosis may very well have "concealed itself" under the complex of symptoms called "wilt disease of carnation", which is well known in this country and the etiology of which has not been clarified so far. In June–July 1956 the author, however, carried out a comprehensive pathological analysis of altogether 53 Danish carnation nurseries, and thereby he found that out of 147 clones (stocks of plants) examined, 39—distributed in 10 different varieties, or 26.5 $^0/_0$—were infected by bacterial stunt of carnation (cf. Tab. XV, p. 147).

S y m p t o m s a n d e t i o l o g y. The bacterial stunt of carnation attacks solely the vascular tissues of the carnation stems. The vessels are choked and decomposed thereby, and in malignant cases the bacteriosis may thus form tubular cavities in the stems. The infection very often causes a stunted growth of the young shoots, but the pathogen of bacterial stunt of carnation, however, does not seem to produce so much growth-stimulating substance that the carnation—as in the case of bacterial stem-crack—reacts by such vivid callus formation round the attacked tissue portions that the stems will crack. The attacked carnation will, however, attempt, by some production of meristematic cells, to stave off the bacterial attack, and this results only in a more or less pronounced thickening of the stems. If cracks occur, they are as a rule small and strictly superficial, and so far the author has seen no bacterial slime in them at all. The bacteria, therefore, have no or very little chance of being

transferred from diseased carnation stems to healthy ones, but as the bacteria are present in abundance in the infected vessels, the young shoots—the cuttings—of the carnation plants will often contain bacteria in the vascular tissues also. Therefore, like bacterial stem-crack of carnation, bacterial stunt of carnation is transferred by infected cuttings taken from diseased mother plants. If the infection is extensive, the cuttings will fail to form roots while standing on the propagation bench, and they will wilt with a non-characteristic, greenish-grey discoloration very reminiscent of withering-up due to lack of water. By cutting up the wilted cuttings it is found that numerous vessels have a brownish-yellow discoloration and are full of bacterial slime, and in several cases the lowermost part of the stem is soft rotted.

If, on the other hand, but few vessels are invaded, the cuttings will as a rule form some roots and start growing to some extent, but the young shoots then become characteristically short and thick—the internodes are shorter than normal (Figs. 20 & 21)—and moreover the leaves of the shoots become somewhat narrower than usual. These are the symptoms which have inspired the author to propose the name: "bacterial stunt of carnation".

The further course of the disease after the rooting period will depend to some extent on the treatment of the plants. In most cases, however, the carnations of relatively feeble growth will not stand transplanting and therefore wilt rapidly after this process. Plants which are attacked only in a slight degree are better able to stand planting-out, and so they will often grow normally for a long time—even for many months. By closer inspection of the crop it will no doubt be found that some of the plants—viz. the infected ones—are a little shorter and of a denser growth (stunted shoots) than the other, healthy plants in the bed. In the attacked carnations it may also be observed that some of the stems are thickened at the bases and have cracks here and there, although these cracks do not occur so frequently and are just of a superficial nature, quite different from those caused by bacterial stem-crack of carnation. Nor is sticky bacterial slime observed such as is found in the case of the latter disease.

Primarily infected plants, i. e. plants which have been infected in some way or other before transplanting at their final growing place, will as a rule never become more than six to twelve months old and thus about 50 to 100 cm tall. The healthy carnations will then outgrow the diseased ones, which are left as lower plants among the healthy ones, and sooner or later the attacked plants will wither, while turning greenish-grey.

Fig. 20. "William Sim", artificially infected (inoculation at "A") with *Pecto-bacterium parthenii* var. *dianthicola*. Photographed 4 months after inoculation (2.1.1956) with isolate No. 98. Note the left-hand, much stunted, shoot; the right-hand one has not yet become visibly infected. ×0.5.

without any other outward sign of the attack of the disease than the altogether exceptionally short and dense shoots. The root system of the plants is often comparatively well preserved, and most of it will generally follow the plant when uprooted. When the stems are cut lengthwise, few or several long, brownish stripes are seen in the xylem, originating from bacteria-filled and discoloured vessels; and when the stems are cut transversely, the discoloured vascular tissues appear as more or less irregularly formed and spread brownish-yellow blotches on the cut-surface (Fig. 39, p. 168); the margin of the blotches, furthermore, has very often a somewhat "water-soaked" appearance.

The roots of younger, attacked plants seem as a whole to be unaffected at the early stages of the disease, but the few roots having direct connection with the infected vessels are often brown and in a decomposed state, and no doubt the dissemination of the disease in 1- or 2-years-old stocks of carnations springs from there. Where the disease is more advanced, however, the roots of the plants may be found to have rotted away completely.

Secondarily infected plants, i. e. plants which have been infected in the transplanting bed, will often wilt suddenly—if heavily infected—, assuming a greenish-grey discoloration of all hitherto green parts. But as a rule, there is no time for these tall plants to form short shoots, and in the early stages of the disease the larger proportion by far of the roots of the plants is healthy. Only part of the upper roots of the system suffer from soft rot, and there is therefore every probability that the bacteria invade the older plants through these roots. Whether the roots are primarily killed by the bacteria or the latter penetrate into roots already killed or weakened in some way or other, has been impossible to ascertain, for one thing because it is impossible to provide unwounded cuttings.— It is a fact, however, that wounded roots provide excellent entrance for the bacteria, and it is hardly imaginable that older carnation plants would not have a number of dead or dying roots so that it always would be easy for the infection to be established through them. Finally, it may be taken for granted that the pathogen of bacterial stunt of carnation—in analogy with other species of *Pectobacterium*—is a pronounced wound parasite.

In secondarily infected plants the bacteria are generally observed not very high up the stems; (5)–20–40 cm is common, but these lower parts of the stems may often be heavily infected. However, the risk of taking infected cuttings from older, secondarily infected plants may not always be great. These plants are usually so tall that it can hardly be a question

Fig. 21. "William Sim", artificially infected (inoculations at "A"), partly (the plant to the left) with *Pectobacterium parthenii* var. *dianthicola* (isolate No. 98), partly (to the right) with *Pseudomonas caryophylli* (isolate No. 83). Note the stunted shoot of the plant to the left and the normal shoots and deep stem-cracks of the plant to the right. Photographed 1.11.1955. × 0.3.

of taking cuttings from the lower parts of the stems, which the bacteria may have invaded; and the plant will generally die before the bacteria reach the top. On the other hand, if carnations are slightly—primarily or secondarily—infected the bacteria will be found also in the upper parts of the plants. Such plants will grow for a long time with an appearance which is hardly distinguishable from that of healthy plants, and as the disease in these plants with few infected vessels is furthermore found considerably higher up the stems (often right at the top), the risk of taking infected cuttings will be particularly great with such "healthy carriers of infection".

Contrary to bacterial stem-crack, bacterial stunt of carnation seems to have a great tendency to spread in older carnation stocks, and in the course of a year's time it can kill so many plants in the beds that the well-known gaps arise, which may be of very different sizes; 1–5–10 m is not unusual (cf. Fig. 36, p. 156). The author has tried to find the cause of the occasionally almost epidemic dissemination (anything but "slow wilt"!) of bacterial stunt of carnation in older stocks—a thing which in comparison with the essentially more extensive root destruction of bacterial stem-crack would not appear to be self-evident. It would thus be reasonable to expect that a larger proportion of rotted roots would also give more bacteria in the soil and thereby afford a greater chance of attack. This presupposes, of course, that the edaphic factors and the ways of infection are alike for the two diseases, but that has apparently been the case in the nurseries concerned.

As far as the author is able to judge (no analysis has been made for determining the content of bacteria in the soil), the different conditions of dissemination of the bacterioses may be accounted for by, inter alia, the widely different temperature requirements of these two bacterioses. The optimum temperature for the *Pectobacterium* species is about 25–27°C, but this bacterium will grow very well at lower temperatures, say, 10–15°C. The temperature requirement of *Pseudomonas caryophylli* is, however, considerably higher, the optimum temperature being about 30–37°C, and this bacterium grows extremely poorly at 15°C. It must therefore be very difficult for *Pseudomonas caryophylli* to grow in Danish carnation beds, in which the soil temperature is most often below 10–12°C; in the coldest months of the year the temperature is even as low as 5–6°C, but in the warmest, brief, spells it may rise to about 18–20°C. However, the pathogen of bacterial stunt of carnation will grow excellently at these temperatures. Furthermore, this bacterium will also grow well on various plant parts; for instance, it grows well on both

raw carrots and raw potatoes, which are decomposed completely; on these media *Ps. caryophylli* will grow only with difficulty or not at all. These facts tend to indicate that the new carnation pathogen is capable of living saprophytically in the soil in a higher degree than *Ps. caryophylli*, and the growth and dissemination of the bacteria are undoubtedly also promoted thereby.

Altogether, the pathogen of bacterial stunt of carnation seems to be better adapted to the cool climatic conditions prevailing in Denmark than is that of bacterial stem-crack of carnation, which in this country seems to be at the northern limit of its habitat.

3. Isolation of the Pathogen

When bacterial stunt of carnation was observed for the first time in Denmark, in cuttings of the variety "Salmon Sim" and in mother plants of "Lucky Strike", this disease was, as already mentioned, first supposed to be an attack of bacterial stem-crack of carnation (*Pseudomonas caryophylli*). But in order to make sure of the diagnosis, an isolation from the vascular tissue with brownish-yellow discoloration was attempted. For both varieties, the isolation was done so that about a dozen plants showing signs of incipient wilting were selected and the lowermost 5–10 cm of the stems were cut off and discarded. The cut-surfaces of the stems were then examined through a hand lens, and five of the plants most feebly attacked, i. e. plants in whose transverse section of stem but few and small brown spots were found in the xylem, were selected for the final isolation tests. The leaves of these cuttings were removed, and the stems were rubbed hard and washed carefully with "oxyspirit" (70 $^0/_0$ ethanol + 1 $^0/_{00}$ 8-hydroxy-quinoline sulphate) and, finally, flamed slightly. Thereafter the lowermost portion (2–3 cm) was cut off the stems and discarded, and after the upper boundary zone between the healthy tissue and the discoloured vascular tissue had been selected, small pieces of tissue were aseptically cut out (with a "Gillette" scalpel No. 3) from the discoloured vascular tissue and transferred directly into a few millilitres of sterile, distilled water in test tubes. After 4 hours at room temperature, it was possible to demonstrate, by microscopy, numerous bacteria in the water, which was therefore streaked on to, potato dextrose agar (PDA) as well as on nutrient agar, in Petri dishes (10 cm × 1 cm); and for each of the five suspensions one dish of each medium was used, i. e. 2 × 5 = 10 dishes in all. These were incubated at 27°C, and after 24 hours it was possible to distinguish

an incipient growth of bacteria on both media. This was already suspicious in itself, since *Ps. caryophylli* as a rule did not appear until after about 2 days. After 5 or 6 days it was evident that *Ps. caryophylli* was not present on the agar plates, its highly characteristic dark-brown, smooth-margined colonies being not at all found. In all the dishes there appeared, however, some roundish, slightly convex and quite faintly greyish colonies, which after 2 or 3 days had developed characteristic, lobate margins, which eventually became quite coralloid (Figs. 24 & 25, pp. 112 & 113). The centres of the colonies became conspicuous with age, particularly in transmitted light, as distinctly brownish-yellow, slightly raised (umbonate) projections. In most of the dishes this type of colony was found in a perfectly pure population, and as the department's greenhouse and incubator capacity did not permit working with several isolates, it was decided to isolate only a single, random isolate, No. 98, from the variety "Lucky Strike", for closer investigation.

4. Infection Tests with the Isolated Bacterium

A. Infection Tests with Rooted Cuttings

a. Inoculation of Stems

With the object of finding out whether isolate No. 98 would be capable of producing disease of any kind in carnations, an infection test was carried out, in which 20 plants of each of the varieties "Lucky Strike" and "William Sim" were used. The test plants were well rooted cuttings, about 8 weeks old, originating from mother plants which had been found by "nutrient broth testing" to be perfectly healthy. The test was run so that 10 plants of each variety were inoculated with bacterial slime of isolate No. 98, taken from a 2-day-old PDA culture. The other 10 plants of both varieties were inoculated with sterile water, and at last all 40 plants were placed in a greenhouse where the temperature was somewhat too high, viz. 20–28°C; it was, however, impossible to obtain a lower temperature in that place.

The actual inoculation was carried out in the way described for bacterial stem-crack of carnation, viz. by piercing in one of the lower internodes on the stem of each test plant three tiny, closely spaced holes into which bacterial slime from isolate No. 98 was smeared directly (cf. Figs. 20 & 21, pp. 101 & 103). The holes were pierced in the stems with a sharp-pointed, flamed needle and roughly made so deep that the bacteria came into direct contact with the vascular tissue.

After inoculation, the test plants were left at rest for 80 days, when the first signs of abnormal growth became noticeable, but hence it was quite plain that the carnations inoculated with bacteria were attacked by a disease causing the plants to develop small, dense shoots with short internodes (Figs. 20 & 21).

When a few of the test plants were cut up, the typical destructions of vessels were refound almost throughout the length of the stem. The rest of the diseased plants were left standing for further observation, and withered up in a month or so. This pathological picture was highly reminiscent of the appearance of the first diseased "Lucky Strike" plants. Re-isolation was made in the manner previously described, from the discoloured upper parts of the vascular tissue, and the appearance of all the bacterial colonies produced thereby was in perfect agreement with the original isolate No. 98 (Fig. 24, p. 112). From among the re-isolates a single one (No. 99) was picked for the re-inoculation, which was carried out as described above, and which resulted in the occurrence of short shoots in the inoculated plants. The plants wilted in 2 or 3 months, while assuming a greenish-grey discoloration; all the check plants stayed healthy for over 6 months and were then discarded.

Hence it might be taken for granted that the bacterium isolated from "Lucky Strike" was really an organism which was pathogenic to carnations and capable of making perpetual-flowering carnations wilt with such symptoms that the disease could easily be confused with bacterial stem-crack.

Meanwhile, pure cultures of apparently the same species of bacterium isolated from stocks of carnations in Holland (isolate No. 254 from BAKKER & SCHOLTEN) and England (isolate No. R.10 from LELLIOTT) had been received, and with these isolates a large-scale comparative infection test was then carried out, comprising also the Danish original isolate, No. 98, and the re-isolate, No. 99. Furthermore were included two Danish *Erwinia* isolates from potato tubers; one of them (No. 35) had been determined by HELLMERS & DOWSON (1953) as *Erwinia carotovora* (JONES) HOLLAND and the other (No. 25) as *Erwinia atroseptica* (VAN HALL) JENNISON.

For each of the 6 isolates, 10 plants of the variety "Peppermint Sim" and 20 check plants (inoculated with sterile water) were used, that is, altogether 80 plants originating from healthy mother plants and about 6 to 8 weeks old. As an extra check on the test, also 10 more plants were inoculated with isolate No. 83 (the Danish original isolate of *Pseudomonas caryophylli*).

The inoculations were performed in the usual way, by streaking bacterial slime from 2-day-old PDA cultures into stab wounds in the lower internodes; each set of plants was immediately placed in a separate, thoroughly disinfected plant box on a bench in a greenhouse. The test was started in November 1955, and the first 4 or 5 winter months a temperature as low as 2–10°C prevailed in the greenhouse; not until April–May was the temperature raised to 15–20°C, and it was quite impossible to trace anything abnormal about the test plants until altogether 6 months had passed. After that time, however, all the test plants inoculated with the new carnation pathogen (isolates Nos. 254, R.10, 98, & 99) began forming fresh, stunted shoots, with leaves slightly narrower than normal. No difference in pathogenicity was noticeable either in the Dutch, English or Danish isolates, or in the symptoms they produced in the carnations. The plants which had been inoculated with *Ps. caryophylli* displayed bacterial stem-crack of carnation on the lower internodes.

Apart from the plants inoculated with *Erwinia carotovora* (No. 35) and *Erwinia atroseptica* (No. 25) as well as the 20 check plants, which all stayed healthy for over 8 months, all the other plants, which had been inoculated with the new carnation pathogen, showed very great uniformity in respect of the way they wilted and the time it took before they were all killed, which happened in the course of 8 months in all after inoculation. Later infection tests proved that isolates Nos. 25 and 35 had lost their pathogenicity towards potato stems, so the failure of these infection tests with carnations does not of necessity mean that these bacteria are incapable of attacking carnations.

b. Inoculation of Roots

As the new bacteriosis resembled bacterial stem-crack of carnation in several points, it was considered to be of interest to find out whether, like bacterial stem-crack, it was able to infect carnations through their roots. This infection test was carried out so that 40 well rooted carnation cuttings of the variety "William Sim" were first divided into four lots of 10 plants each, and one of these lots (a) was then potted in autoclaved soil and pots to serve as checks. Lot (b) was potted in soil in which the "Lucky Strike" plants attacked by the new bacterial disease had grown 2 months earlier, and both of the last two lots (c & d) were planted in sterile soil and pots; before planting, the plants of lot (c) were, however,

dipped in an aqueous suspension of a mixture of isolates Nos. 98 and 99 cultivated on PDA in test tubes. In the last lot (d) one side of the root system was wounded severely by depression of an autoclaved garden label near the stem immediately on being planted in sterile soil; into the hole thus produced a quantity of a bacterial suspension similar to the one used for the dipping of lot (c) was poured. All 40 plants were placed in new plant boxes on benches in a greenhouse, in which a temperature of about 15–30°C was prevailing most of the time; and in the following time these plants received normal care and attendance.

This test proved fully that infection can take place through the roots, since both of the lots (c & d) which had been treated with aqueous bacterial suspensions of a mixture of isolates Nos. 98 and 99 developed stunted shoots in 2 or 3 months and wilted in another month or so. Only 4 of the plants which had been planted in bacteria-containing soil (b) became diseased; the rest and all the check plants (a) stayed healthy.

B. Inoculation of Rootless Cuttings

The practical experience of bacterial stunt of carnation is that the cuttings, as in the case of bacterial stem-crack, may be widely infected during the stay on the propagation bench. This was also borne out by a small infection test performed in the way that 30 healthy ("nutrient broth tested") cuttings of the variety "Lucky Strike" were inserted in an entirely new plant box with autoclaved sand. In the middle of this box also 10 cuttings of the variety "William Sim" were placed, which were known with certainty to be attacked by bacterial stunt of carnation. The cuttings were set in sand in mid-December 1955 and left in the aforementioned, too cold, greenhouse over the winter.

The 10 cuttings already infected all wilted in 2 months, but a couple of them formed a few roots. The other plants began developing fresh shoots after 4–5 months, but 6 of the plants—those standing close beside the diseased cuttings—formed the characteristic stunted shoots, and from these plants the pathogen of bacterial stunt of carnation was isolated. The rest of the plants stayed healthy, as did some check plants which had been set in sand in a separate box. Unfortunately, the test was made under very adverse conditions in the cold greenhouse, and the cuttings were therefore left in the sand for a very long time, but there was no doubt that the bacteria were able to spread from the diseased cuttings to the healthy ones.

Fig. 22. *Pectobacterium parthenii* var. *dianthicola* var. n. Electron-micrograph shaded with palladium. (In making the preparations for the electron-microscopy it appeared to be essential to suspend the 24-hour-old nutrient agar culture in nutrient broth and not to fix the bacteria with formalin before filtration through the collodion film. See also footnote p. 36). Phot. A. BIRCH-ANDERSEN, M.Sc., Statens Seruminstitut, Copenhagen, Denmark. ×23000.

5. Description of the Isolated Bacterium

A. Morphology and Staining Reactions

The isolated bacterium is Gram-negative, rod-shaped with rounded ends, and has 1–12 peritrichously arranged flagella (Figs. 22 & 23); LELLIOTT indicates 3–14, though most often 8–11, but the present author believes that these figures are somewhat too high. By cultivation on nutrient agar, LELLIOTT found that the bacterial cells measured 0.8–3.2 × 0.5–0.8 μ; this is in good agreement with the measurements (0.7–3.5 × 0.5–0.9 μ) found by the present author for bacteria cultivated on PDA and measured in Congo red. The bacterium produces no spores and generally occurs singly or in pairs; now and then it may, however, form long chains, especially in liquid media.

The bacterium stains readily with the common dyes and methods;

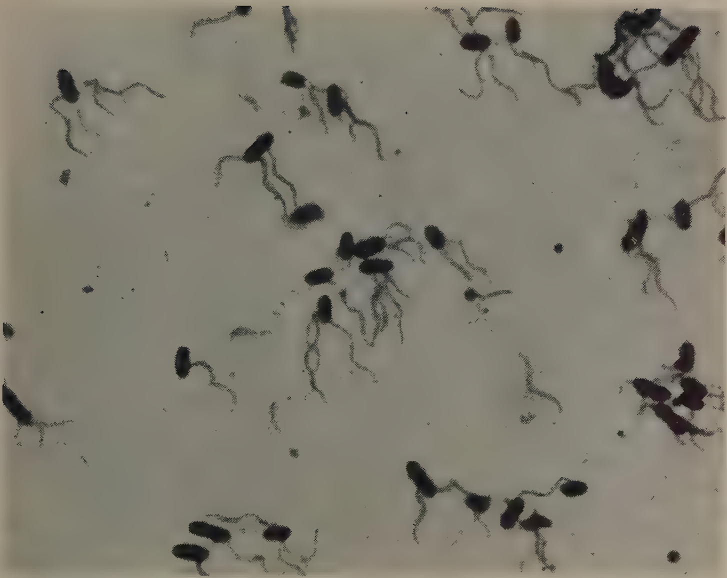

Fig. 23. *Pectobacterium parthenii* var. *dianthicola* var. n., stained according to MARTIN KRISTENSEN's "silver-sulphate method" (1955). ×2000.

e. g. carbol fuchsin; VILHELM JENSEN's (1931) mordant and also MARTIN KRISTENSEN's (1955) modification of ZETTNOW's so-called "silver- sulphate method" (Fig. 23) were found to be excellent flagella stains.

B. Cultural Characters on Different Media

Nutrient agar (Difco) is an excellent medium for this bacterium, which after 24 hours at 26–27°C forms circular, slightly convex and dirty-white, granular, shiny colonies with a somewhat lobate margin. After 2 days, the colony has a slightly lobate margin and resembles closely cultures of *Erwinia* (*Pectobacterium*) *carotovora*. On this medium the bacterium is able to keep alive for about 1 or 2 months at ordinary room temperature. If the culture is covered with medicinal paraffin oil, it may subsist for more than 6 months.

Nutrient broth (Difco). The bacterium grows excellently in this medium, which becomes highly turbid in 12–24 hours, and positive transfers can be made even after 2 or 3 months.

Potato dextrose agar (PDA) (Difco). At 26–27°C the bacterium

Fig. 24. *Pectobacterium parthenii* var. *dianthicola* var. n., a 10-day-old pure culture, No. 98, on PDA. Note the lobate edge of the colonies (see also Fig. 25). Photographed 17.12.1955. ×0.7.

forms small, circular, dirty-white, granular colonies in 24 hours, and after 2 or 3 days the centre of these colonies may assume a very faint, brownish-yellow hue; this becomes more intensely brownish-yellow at the middle of the culture and appears there as a more or less sharply defined light brown spot, which becomes somewhat umbonate with age. Already after the first few days have passed, the margin of the colony is distinctly lobate, and in certain cases it may be almost coralloid (Figs. 24 & 25). LELLIOTT writes in his diagnosis that young (4- or 5-days-old) cultures may resemble fried eggs, a comparison which the author finds very appropriate. Bacterial colonies on PDA become firm with age (1 or 2 months), so that it is difficult to scrape off bacterial slime for transfers, and the bacterium will hardly survive more than 2 or 3 months' storage at ordinary room temperature; when covered with medicinal paraffin oil it may be kept alive for 6 months. Cultures on PDA have a very distinct smell resembling, if anything, that of boiling soap-suds. The smell is so characteristic that it can be used as a diagnostic factor.

Sterile potato plugs are completely dissolved in 1 or 2 days at 26–27°C; the whole "mass" assumes a brownish-grey hue.

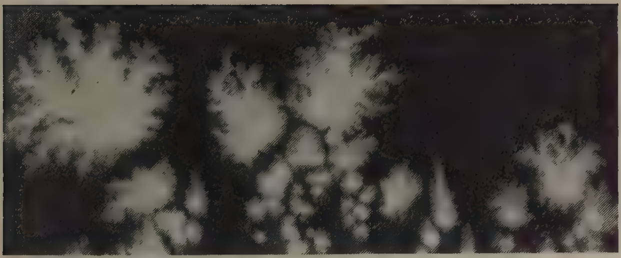

Fig. 25. Colonies of *Pectobacterium parthenii* var. *dianthicola* on PDA. ×2.5. Note the coralloid edge.

Nutrient gelatin (Difco) is liquefied. At 22°C the volume liquefied after 2 or 3 days is somewhat napiform at the top, but subsequently it becomes saccate, and after 8–14 days all the gelatin (6 ml) in the test tube is liquefied.

Endo agar (Difco). The bacterium grows readily on this medium, and in 1 or 2 days it forms pink colonies which—like the medium—preserve this colour throughout 3 weeks' storage at 26°C.

Eosin methylene blue agar (E.M.B. agar, Difco). On this medium the bacterial colonies (at 26°C) are reddish and mucoid, and many "violet spots" (probably of an eosin methylene blue compound) (Wynne et al. (1942)) are visible in the bacterial slime. The medium does not change its colour.

Desoxycholate agar (Difco) was employed with a covering layer of the medium and thereby gave the most reliable results. With this method, and at 26°C, the colonies are at first quite faintly yellow and later palely pink. The medium does not change its colour, or it becomes slightly paler.

In sterile (autoclaved for 3 hours), moist garden soil at 10°C the bacterium is kept well alive for two months, but it could not stand 4 months storage.

C. Influence of Temperature on Growth of the Bacterium

The temperature requirement of the bacterium was determined by inspection of PDA and nutrient agar cultures inoculated with a suspension of a 2-day-old nutrient broth culture of isolates Nos. 98, 99, R.10, and 254[1]). The test tubes with the cultures were incubated at 2, 5, 10, 15, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, and 36°C, respectively, and

[1]) Cultures R.10 and 254 are the English and Dutch isolates, respectively.

after 1, 2, 3, and 6 days the bacterial growth on the media was checked. In this way the optimum temperature was determined to be 25–27°C; above this temperature the growth was inhibited somewhat, and at 35–36°C it took 6 days before bacterial growth could be detected on the media. At 2°C no growth was visible on the medium, but at 5, 10, and 15°C it was substantial after 8–9 days. The bacterium will stand storage for 4 months on nutrient agar at minus 6°C. The thermal death point was determined by 10 minutes' heating of one ml nutrient broth culture (24 hours old) and immediate transfer to fresh nutrient agar, and was found to be between 49 and 50°C.

D. Biochemical Reactions

The ability of the bacterium to ferment carbohydrates, alcohols, glucosides, and sodium salts of some organic acids was checked by cultivation in a liquid, inorganic medium (for composition, see footnote on page 39), to which was added $1^0/_0$ of the carbon compound in question the sodium salts, however, only in $0.15^0/_0$. Bromthymol blue was used as indicator, and the hydrogen-ion concentration of the medium was adjusted to pH 7.2. The media were autoclaved 10 minutes at 110°C. After 1, 2, 3, 6, 9, and 20 days at 26–27°C in the incubator, acid had been formed (the colour of the medium changed from greenish-blue to yellow) and gas was formed in the tubes with arabinose, xylose, glucose, galactose, mannose, fructose, saccharose, raffinose, inulin, mannitol, and salicin. Rhamnose, glycerol, aesculin, and ethanol were also fermented with production of acid, but no formation of gas was noticeable. Lactose was fermented in 3 weeks; maltose was not fermented in this period, or at any rate only extremely feebly. In the tubes with dextrin, erythritol, sorbitol, dulcitol, Na-hippurate, and NH_4-lactate some growth was observed, but after 3 weeks none of the media had changed their colour, and the ability of the bacteria to ferment these carbon compounds may therefore be considered negative. In Na-acetate, Na-citrate, Na-succinate, Na-tartrate, and Na-malonate alkali was produced. Nutrient broth with $1^0/_{00}$ aesculin added was coloured black in 1 day (cf. Table XII, p. 122). No growth was found in nutrient broth + $5^0/_0$ NaCl.

The diastatic reaction of the bacterium was investigated by cultivation on nutrient agar plus $0.2^0/_0$ water-soluble starch, but after 2 and 4 weeks, respectively, none of the starch had been hydrolyzed, as demonstrated by testing with Lugol's liquid.

A slight growth was noticed in Fermi's solution, and by cultivation in Difco nutrient broth it was not possible after 2, 4, 8, and 16 days to notice any formation of ammonia (test with Nessler's reagent). By cultivation in Difco nitrate broth a distinct formation of nitrite was manifest already after 1 day (test with Gries's reagent).

The test for indole formation was positive; it was made by cultivation on a trypsin-casein solution and on a 1⁰/₀ Difco bacto-tryptone solution with the addition after 6 and 9 days of a few drops of para-dimethylaminobenzaldehyde dissolved in amyl alcohol and concentrated hydrochloric acid. The reagent was red-stained.

The hydrogen sulphide test was negative. For this test the bacterium was cultivated on Difco iron agar, but after altogether 4 weeks no brown-staining of the medium could be traced. Lead acetate paper above cultures grown on PDA, nutrient broth and sterile potato plugs gave no positive result either.

The methyl-red test was positive after 2 days, but after 5–6 days it became negative. The Voges-Proskauer test was positive all the while (cultivation on Difco M.R.-V.P. medium).

Neutral litmus milk (Difco) was turned into soft curd in 6–8 days, and simultaneously a clearing (peptonizing) of the top layer (about 0.5 mm) of the medium started; after 2–3 weeks the curd was firm. After 3–4 days the medium assumed a bluish-grey colour, but litmus was reduced after 6–8 days, the medium assuming the colour of light clay. After 3 weeks the colour did not change, and the clearing which had begun did not proceed deeper into the medium.

In all the cultivation tests two Danish carnation isolates, Nos. 98 and 99, as well as one Dutch, No. 254, and one English, No. R.10, were included; for all 4 isolates, 2 tubes of each medium were used. The cultivation tests were repeated 3 times, but no difference could be observed from time to time or between the different isolates. The biochemical reactions of the four carnation isolates and their cultural characters are thus not only in close inter-agreement but are also in complete conformity with LELLIOTT's description (1956) of the English isolates, of which one (R.10) was also included in the author's cultivation tests. There is thus good reason for considering the four isolates perfectly identical, and hence it follows that the disease occurring in stocks of English, Dutch and Danish carnations must be the same.

E. Comparative Infection Tests with Carnation Isolates and Isolates of *Erwinia carotovora*, *E. atroseptica*, *E. chrysanthemi*, and *E. carotovora* f. sp. *parthenii*

After the biochemical reactions and cultural characters of the "carnation organism" had been analysed, also its pathogenicity towards potatoes, chrysanthemums and carnations compared with related species of bacteria was investigated.

For comparison with the four carnation isolates (Nos. 98, 99, R.10, and 254), the tests included an isolate of *Erwinia carotovora* (Jones) Holland (isolate No. 35 from a Danish potato tuber ("Bintje")), an isolate of *Erwinia atroseptica* (van Hall) Jennison (isolate No. 25 from a Danish potato tuber ("Up-to-date")), an isolate of *Erwinia chrysanthemi* Burkholder et al. (isolate No. 188 from ATCC No. 11662), and an isolate of *Erwinia carotovora* f. sp. *parthenii* Starr (American isolate from M. P. Starr No. E. c. 110).

For this test, the abovenamed eight isolates were first cultivated for two days on PDA in test tubes, whereupon the bacteria were transferred with an inoculation needle into 3–4 stabs in the lowermost internode of about 2-month-old carnation plants of the variety "White Sim". For each isolate 10 plants were used, and altogether 20 plants were inoculated with sterile water to serve as checks. The test plants were placed in a greenhouse immediately after inoculation, and the following months they were tended in the usual way.

In 1–4 months the plants inoculated with carnation isolates formed young shoots with characteristically short internodes. By random sampling from the stunted plants, bacteria identical with the inoculate were isolated consistently.

In this period none of the carnations which had been inoculated with the other *Erwinia* isolates displayed any symptom of stunted shoots. However, this need not mean that the isolates are non-pathogenic, since it is necessary to await incubation for at least 12 months. After 4 or 5 months the plants had, however, suffered so badly by their stay in the greenhouse, which was extremely unfit for the growing of carnations, that the test plants had to be discarded, and continued long-term inoculation tests were considered impracticable.

Concurrently with the infection tests on carnation, all 8 isolates were inoculated on stems of potato plants ("Bintje") and *Chrysanthemum indicum* ("Mayland Flame"). In total, 5 stems of each species of plants were used per isolate, and an equal number of stems were inoculated with sterile water for checking.

The potato plants were inoculated on the method described by HELLMERS & DOWSON (1953), by introducing bacterial slime through a minute cut, directly into the big vascular bundles behind the so-called "wings" on the stems. Like the carnations, the chrysanthemums were inoculated by streaking bacterial slime into stabs in the stems. Both species of plants were placed in a humid atmosphere, in "Hansen Boxes" or covered with plastic bags, in a greenhouse. In 2–4 days all the stems of the potatoes and the chrysanthemums which had been inoculated with carnation isolates, *Erwinia chrysanthemi*, and *E. carotovora* f. sp. *parthenii*, developed typical "black leg" above and below the stabs, though mostly above them (Figs. 26 & 27).

Even after 14 days, isolates Nos. 25 (*E. atroseptica*) and 35 (*E. carotovora*) were incapable of attacking either stems of carnation, potato or *Chrysanthemum*, and thus, unfortunately, it was found that both of these isolates, which in 1953 had proved very active pathogens by inoculation in stems of potato, tomato and tobacco (HELLMERS & DOWSON 1953), had now completely lost their pathogenicity. It was therefore, furthermore, investigated whether the two isolates were also unable to produce soft rot on slices of raw potatoes and carrots. Simultaneously, the ability of the carnation isolates, *E. chrysanthemi* and *E. carotovora* f. sp. *parthenii*, to produce soft rot on the same media was tested. Each of the 8 isolates was then inoculated on three slices of raw carrots placed on moist filter paper in Petri dishes. In each dish an uninoculated slice was put, in addition, as a check, and all the dishes were left at room temperature (about 18–20°C).

The result of the inoculations was positive, all the isolates, also Nos. 25 and 35, producing a very uniform and typical soft rot. Moreover, it was investigated whether isolates Nos. 25 and 35 were able, after growth on the carrot plugs, to induce "black leg" on potato stems, but these isolates did not produce any infection either.

For lack of a suitable greenhouse, the infection tests reported on in the above had to be carried out in a very unsatisfactory manner. However, the inoculations proved that neither the "*Chrysanthemum*" nor the "*Parthenium*" nor the "*Dianthus*" isolates seemed to be specific in the choice of their hosts.

6. Discussion of the Taxonomy and Nomenclature of the Isolated Bacterium

According to LELLIOTT's description (1956) as well as the author's, the "carnation bacterium" is Gram-negative and rod-shaped with

rounded ends, and forms neither capsules nor spores; it is motile and has 1–14 flagella peritrichously arranged, and it is able to ferment salicin and a large number of "sugars" with the formation of acid as well as gas. Finally, it is a character of this bacterium that it is capable of decomposing living plant cells, e. g. vascular tissue of carnations, carrots and potato tubers, the latter two under development of a pronounced "soft rot". When inoculated on healthy stems of potatoes and chrysanthemums, the bacterium will produce a typical "black leg".

The morphological, pathological and biochemical characters of the carnation pathogen are thus in excellent agreement with the descriptions of the genera *Erwinia* WINSLOW et al. (1917) and *Bacterium* DOWSON (1939). At the Microbiological Congress in Rome in 1953 the latter generic name was rejected and so inapplicable to plant pathogenic bacteria, and the carnation pathogen must therefore in the first place be designated as a "member" of the genus *Erwinia* WINSLOW et al. This genus seems, however, to comprise fairly heterogeneous species, and E. L. WALDEE (1945) has proposed to divide the genus into two so that the generic name of *Erwinia* should be preserved for the non-pectolytic species, e. g. *Erwinia amylovora* (BURRILL) WINSLOW et al., while the pectolytic species should have the generic name of *Pectobacterium*; and as type species for this genus WALDEE chose *Erwinia carotovora* (JONES) HOLLAND, which was hence called: *Pectobacterium carotovorum* (JONES) WALDEE. WALDEE's views are also adopted by W. J. DOWSON (1957).

In accordance with the above description of the carnation pathogen, this must be classified as a typical "member" of the genus *Pectobacterium*, and as the author—like W. J. DOWSON—considers WALDEE's division of the genus *Erwinia* both well founded and practical, it would appear to be natural to refer the new carnation pathogen to the genus *Pectobacterium* WALDEE.

Whereas the genus determination of the "carnation bacterium" is relatively satisfactory, its species determination seems to be very complicated. LELLIOTT (1956) in his preliminary description of the bacterium states that it must be considered a strain of *Erwinia chrysanthemi* BURKHOLDER et al. (1953), but he has not thereby given any specific name to the "carnation strain". The author of this paper has therefore tried if it would be possible to provide a more exact characterization of the carnation isolates and thereby find an appropriate name, and with this end in view a long series of cultivation tests have been carried out with the undermentioned 12 isolates[1]) (species and strains):

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[1]) The banana isolate (S. 9) has been used in a few tests only.

Fig. 26. *Chrysanthemum indicum* ("Mayland Flame") artificially infected with *Pectobacterium parthenii* var. *dianthicola*, isolate No. 98, photographed 10 days after inoculation (at "a"). Note the wilted top shoots and the black-staining of stem to "b" as well as of the basal portion of the first leaf below "a". ×0.5.

No.			
98:	Carnation isolate,		the author's original isolate.
99:	–	–	the author's re-isolate.
R.10:	–	–	Lelliott's isolate from English carnations. Isolated 1955.
254:	–	–	Bakker & Scholten's isolate from Dutch carnations. Isolated 1956.
35:	*Erwinia*	*carotovora*	(Jones) Holland, the author's isolate from a Danish potato tuber ("Bintje"). Isolated 15.1.1951.
904:	–	–	(Jones) Holland, W. H. Burkholder's isolate (E.34) from *Iris* pods.
25:	–	*atroseptica*	(van Hall) Jennison, the author's isolate from a Danish potato tuber ("Up-to-date"). Isolated 13.9.1949.
188:	–	*chrysanthemi*	Burkholder et al., American isolate from ATCC No. 11662.
E.c. 17:	–	–	Burkholder et al., American isolate from M. P. Starr.
E.c. 18:	–	–	Burkholder et al., American isolate from M. P. Starr.
E.c.110:	–	*carotovora* f. sp. *parthenii*	Starr, American isolate from M. P. Starr,
(S.9:	–	sp., banana isolate from M. P. Starr.)	

In this review and the subsequent discussion the author has chosen to retain the generic name of *Erwinia*, partly because the isolates were received under this name and partly because reference in this section to the genus *Pectobacterium* would involve very complicated names, making the discussion even more cumbersome.

Each isolate was first subcultured on nutrient broth (Difco) in 4 test tubes, and after 1 day's growth at 26°C transfers were made from these tubes to two tubes (or Petri dishes) of each of 44 bacteriological media. As far as the carbon compounds are concerned, these media were produced on the basis of an inorganic synthetic medium, as indicated on page 114, and using bromthymol blue as indicator; the other media were made from Difco preparations. All the inoculated media were immediately incubated at 26°C, and after 1, 2, 3, 6, 9, and 20 days, respectively, records were made of the cultural characters and biochemical reactions. The results of the cultivation tests, which were repeated 3 times, with the same result, are listed in Table XII.

It may be seen from Table XII that the 11 isolates react uniformly when cultivated on or in the following 30 out of the altogether 44 media: arabinose, xylose, rhamnose, glucose, galactose, mannose, fructose, maltose, saccharose, raffinose, dextrin, starch, glycerol, erythritol,

Fig. 27. *Solanum tuberosum*, "Bintje", artificially infected with *Pectobacterium parthenii* var. *dianthicola*, isolate No. 98, photographed 20.6.1956, 6 days after inoculation. The two inoculation points are wrapped with "Cellotape". The inoculated stems became soft-rotted, and black-stained, and broke. On the stem to the left the black-staining has not yet reached the top and it therefore appears most plainly on this stem. ×0.3.

Table XII. Review of biochemical reactions of various types of isolates:

"*Parthenium*" isolate (No. E.c. 110) .... P
"*Chrysanthemum*" isolates (Nos. E.c. 17, E.c. 18, 188) C
"*Dianthus*" isolates (Nos. 98, 99, R.10, 254) .... D
"*carotovora*" isolates (Nos. 35, 904) c
"*atroseptica*" isolate (No. 25) .... a

(Temperature: 26°C)
(Indicator: bromthymol blue)

Medium	Acid[1] (medium yellow)				Growth but no change in reaction			Alkaline (medium blue)		
	After days				After days			After days		
	1–2	3–6	6–9	9–20	1–2	6–9	9–20	3–6	6–9	9–20
Arabinose		all								
Xylose		all								
Rhamnose			all							
Glucose	all									
Galactose		all								
Mannose	all									
Fructose		all								
Maltose					all					
Saccharose		all								
Raffinose		all								
Dextrin							all			
Starch							all			
Glycerol			all							
Erythritol							all			
Sorbitol							all			
Mannitol		all								
Dulcitol							all			
Salicin	all									
Aesculin		all								
Na-citrate									all	
Na-hippurate							all			
Na-succinate									all	
NH_4-lactate						all				
Nutrient agar					all					
Nutrient gelatin						all saccate				
Fe-agar (H_2S)						all				
Fermi's solution					all					
Lactose	c.a.			C.P.D.						
Inulin	C.		D.				P.c.a.			
Na-acetate								C.P.D.		c.a.
Na-tartrate							C.c.a.		P.D.	
Na-malonate							c.a.	C.P.D.		

[1]) The ability of the isolates to form gas has not been taken into account, because it is very inconsistent and, consequently, of minor importance in this connection.

Table XII, continued.

Medium	Reactions						Indol	
	Acid (medium yellow)	Growth	No growth	Alkaline (medium blue)	Medium black	NO_2 produced	+	—
	After days							
	9–20	1–2	20	9–20	1–2	2	9	20
Nutrient broth		all						
– – + 1°/₀₀ aesculin					all			
– – + 1°/₀₀ KNO_3						all		
– – + 5°/₀ ethanol	C.P.D. c.			a.				
– – + 5°/₀ NaCl			D. (P. C. a. c. inconstant)					
Tryptone broth							C.P.D.	c. a.

Medium	Reactions					Growth at	
	Medium pink	Medium deep red	Medium greyish blue	+	—		
	After days						
	3–4	6–8	3–4			35°C	40°C
Endo agar	C. P. D.	c. a.					
Desoxycholate agar	C. P. D.	c. a.					
E. M. B. agar	C. P. D.	c. a.					
Litmus milk	c. a.		C. P. D.				
M. R. after 2 days				C. D.			
– – 6 –					C. D.		
– – constantly				P. c. a.			
V. P. after 2 days					P. C.		
– – 6 –				P. C.			
– – constantly				D.	c. a.		
Nutrient agar						all	C. P.

sorbitol, mannitol, dulcitol, salicin, aesculin, sodium citrate, sodium hippurate, sodium succinate, ammonium lactate, nutrient broth, nutrient broth + 1°/₀₀ aesculin, nutrient broth + 1°/₀₀ KNO_3, nutrient agar, nutrient gelatin, "Fe-agar", and in Fermi's nutrient solution. Moreover, all the isolates were able to grow on nutrient agar at 35°C.

By cultivation on 8 of the media, viz. Endo agar, E.M.B. agar, desoxycholate agar, litmus milk, lactose, sodium malonate, and in sodium acetate, as well as by the indole reaction, the "*Parthenium*", "*Chrysanthemum*" and "*Dianthus*" isolates were found to deviate uniformly from the "*carotovora-atroseptica*" isolates (Tab. XIV, p. 128).

The former 3 isolates can thus be distinguished easily from the "*carotovora-atroseptica*" isolates, but they are difficult to distinguish from each other. It should be observed, however, that the "*Parthenium*" isolate was unable to ferment inulin at all and that the bacterium formed alkali in sodium tartrate after 6 days; excellent growth was found on nutrient agar at 40°C; the methyl red test was constantly positive and the Voges-Proskauer reaction was negative after 2 days, but positive after 6 days at 20–22°C. The "*Chrysanthemum*" isolates fermented inulin with production of acid after 1–2 days, were unable to produce alkali in sodium tartrate, and grew excellently on nutrient agar at 40°C. The methyl red test was positive after 2 days and negative after 6 days at 20–22°C; Voges-Proskauer reaction was negative after 2 days, positive after 6 days. The "*Dianthus*" isolates fermented inulin with production of acid after 6–9 days, produced alkali in sodium tartrate after 6–9 days but were unable to grow on nutrient agar at temperatures above 35°C; the methyl red test was positive after 2 days but negative after 6 days at 20–22°C; the Voges-Proskauer reaction was constantly positive. A review of the different characters of the three types of isolates is given in Tab. XIII.

Thus it would appear that in respect of the biochemical reactions the "*Dianthus*" isolates are more closely related to *E. chrysanthemi* and *E. carotovora* f. sp. *parthenii* than to *E. carotovora* and *E. atroseptica*.

Table XIII. Review of differentiating characters of "*Parthenium*", "*Chrysanthemum*" and "*Dianthus*" isolates.

Medium	"*Parthenium*" isolate	"*Chrysanthemum*" isolate	"*Dianthus*" isolate
Inulin	No acid produced	Acid after 1–2 days	Acid after 6–9 days
Sodium tartrate	Alkaline after 6–9 days	No change in reaction	Alkaline after 6–9 days
Max. temperature for growth on nutrient agar	About 40°C	About 40°C	About 35°C
Methyl red test	Constantly positive	After 2 days positive, after 6 days negative	After 2 days positive, after 6 days negative
Voges-Proskauer reaction	After 2 days negative, after 6 days positive	After 2 days negative, after 6 days positive	Constantly positive

It would therefore immediately be natural to term the "carnation bacterium" a strain of *E. chrysanthemi* or *E. carotovora* f. sp. *parthenii*, or simply consider the "carnation pathogen" identical with the aforesaid bacteria, thereby allowing for minor variations.

However, the determination is by no means as easy as that. Through personal correspondence with M. P. STARR (U.S.A.) the author has learnt that STARR and one of his disciples are at present working out a monograph on the genus *Erwinia* WINSLOW et al., and for the present have arrived at the conclusion—like BURKHOLDER et al. and the author of this paper—that *E. chrysanthemi* (BURKHOLDER et al. 1953) can very well be differentiated from *E. carotovora* on the basis of differences in the biochemical reactions, but that, on the other hand, *E. chrysanthemi* is presumably identical with *E. carotovora* f. sp. *parthenii* (STARR 1947) described by STARR six years before. If this is the case—which already in the description of the *Chrysanthemum* pathogen also BURKHOLDER et al. (1953) considered possible but did not finally establish—, the name of *E. chrysanthemi* must be dropped in favour of *E. carotovora* f. sp. *parthenii*, and consequently the carnation pathogen cannot—according to the rules of nomenclature—be set up as any strain or variety of "f. sp. *parthenii*".

In connection with the abovenamed, somewhat complicated, conditions it may furthermore be observed that at M. P. STARR's they are for the time being also working with a new banana bacteriosis (personal letter Sept. 1956), whose pathogen, it seems, will have to be placed, systematically, very close to the "*Parthenium*", "*Chrysanthemum*" or "*Dianthus*" pathogen.

The difficulties in determining the species of a bacterium within the genus *Erwinia* (*Pectobacterium*) are apparently considerable because our present knowledge of the species characters within these bacteria is very scant, and this knowledge is further depreciated by the fact that the various author's descriptions of one and the same organism—including infection tests—do not seem to be reproducible in several particulars, even when working with the original isolates and recipes for media applied by the investigators concerned.

In order that an attempt may be made at arriving at a usable taxonomy within the group of *Erwinia* species which WALDEE has given the generic name of *Pectobacterium*, the following points of view may be applied:

1. The first point of view is, that *Pectobacterium* isolates from any host plant genus should be set up as new species if only one or a few

reproducible biochemical reactions differ from those of the species hitherto known. From merely plant pathological points of view such a procedure would appear to be attractive: it gives simple names and probably also easily recognisable organisms, but from a point of view of systematics such reasoning is hardly tenable. In recent time, with so many intermediate forms discovered, the tendency seems to be towards preferring to have as few proper species as possible and incorporate in them the several varieties thus coming into consideration.

2. The second point of view that may be adopted in relation to this question of taxonomy is that a few species should be recognized, with allowance for rather considerable biochemical variations.

In this case the carnation pathogen would simply be assumed to be identical with *E. chrysanthemi* or *E. carotovora* f. sp. *parthenii* and be named after either one of these bacteria. But it then has to be decided whether, say, one or three biochemical differences should be recognized as grounds for determining the organism as a species or "merely" a variety, and such judgment would probably lead to so much disagreement and so many non-reproducible results that this "second view" must likewise be considered rather untenable.

3. The third point of view is, that it should be tried to find out, within the genus *Pectobacterium*, groups of types of bacteria which have at least one or two very distinct biochemical reactions in common, so that there may be a possibility of speaking of groups or stirpes.

Within such a stirps a type species is then designated on the usual priority principles, and the rest of the deviating isolate types of the stirps may then be characterised as varieties of the type species, even if determination of these varieties is based only on one or a few—but reproducible—biochemical deviations from the other "members" of the stirps.

The adoption of this reasoning would be more in keeping with modern taxonomic points of view, and at the same time it would be possible to make relatively swift and certain stirps and variety determinations.

In the foregoing, the question of pathogenicity is not touched upon in any of the three reasonings. This has been done deliberately because the author thinks that the heretofore abortive inoculation tests have been carried out under such unsatisfactory circumstances and made the subject of such inadequate studies that they simply cannot be relied upon as a basis for systematic reasoning, and, altogether, pathogenicity may surely be said to be a poor taxonomic character. The application of

"f. sp." within *Pectobacterium* is consequently highly dubious. BURKHOLDER & SMITH's (1949) abortive infection tests with, inter alia, isolate No. 34 (*Erwinia carotovora*) on potato plants, which isolate HELLMERS & DOWSON (1953) four years later found very active towards potatoes, and the author's recent—and unsuccessful—infection tests with isolates Nos. 25 and 35, both of which in 1953 had been highly capable of producing "black leg" in potato plants but had now apparently lost this power, show that both the technique and the age of the isolates call for extremely careful assessment in connection with inoculation tests. However, no mention of such discriminate considerations has been found in the literature available on the *Chrysanthemum* and *Parthenium* pathogens.

In his endeavours to find a useable taxonomy within the genus *Pectobacterium* (*Erwinia*) the author of this paper has tried to stick to "the third point of view" and it really looks as if it would be possible to distinguish between two "stirpes of species" within this genus. One stirps centres about *Erwinia carotovora* and *E. atroseptica* and may here be designated symbolically as the "*carotovora* stirps". It is characterised by producing deep-red colonies of a metallic lustre on Endo agar and by staining this medium dark-red. On E.M.B. agar it forms deep-red colonies without blue-violet "spots" of an eosin-methylene-blue compound (WYNNE et al. 1942), and they are not mucoid ("look dry"). Furthermore, it is characteristic of this stirps that it also forms intensely red colonies on desoxycholate agar and turns litmus milk pink in 3 days, forming firm curd. Lactose is fermented after 1 or 2 days; no indole is formed after 20 days, no alkali is produced in sodium malonate, but after 9–20 days alkali is produced in sodium acetate (Table XIV).

In the other stirps—which symbolically may be called the stirps *parthenii*—it looks as if the pathogens from both *Parthenium*, *Chrysanthemum* and *Dianthus* naturally can be gathered, as they distinguish themselves from the stirps *carotovora* by forming pink colonies on Endo agar without making this medium change its colour; on E.B.M. agar the colonies are redish, mucoid with many "violet spots"; on desoxycholate agar the colonies are very faintly pink or yellowish. After 3 days, litmus milk becomes bluish-grey and is slowly turned into soft curd; lactose is not fermented, or at the earliest after 9–20 days; sodium malonate becomes alkaline after 3–6 days; sodium acetate becomes alkaline after 3–6 days; and indole is produced after 9 days[1]).

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[1]) The banana-isolate may presumably be referred to this stirps, but further tests have not been carried out.

Table XIV. Review of cultural and biochemical differences (at 26°C) between the stirps *carotovora* and the stirps *parthenii*.

Medium	Stirps *carotovora*	Stirps *parthenii*
Endo agar	Colony and medium deep red stained after 6 days	Colony and medium constantly pink
E.M.B. agar	Colony deep red stained, "dry", and no "violet spots"[1])	Colony pink and highly slimy, with many "violet spots"[1])
Desoxycholate agar	Colony deep red	Colony slightly pink or yellowish
Litmus milk	Medium pink after 2–3 days; thereupon firm curd is formed	Medium bluish-grey after 2–3 days; thereupon soft curd is formed
Lactose	Fermented after 1–2 days	Fermented only after 9–20 days, or not at all
Sodium malonate	Medium unchanged or slightly acid	Medium alkaline after 3–6 days
Sodium acetate	Medium alkaline after 9–20 days	Medium alkaline after 3–6 days
Indole	Not produced	Produced after 9 days

[1]) Probably an eosin-methylene-blue compound (WYNNE et al. 1942).

For both stirpes, the abovenamed reactions have been found by cultivation at 26°C. The cultural and biochemical differences between the two stirpes are listed in Table XIV.

The author has chosen, ad interim, the designation: stirps, as a term for the aforesaid two fractions within the genus *Pectobacterium*, but future investigations will presumably change it into either subgenus or species, according to how many and how well separable "members" can be referred to the various stirpes.

There can, however, be no doubt that the three types of bacteria under the stirps *parthenii* are so closely related systematically that they be considered either as identical bacteria or as varieties of each other. But, in any case, the name of "*parthenii*" will have absolute priority within the stirps *parthenii*. The author therefore finds it natural to raise *Erwinia carotovora* f. sp. *parthenii* STARR (1947, p. 299) to a species and simultaneously refer it to the genus *Pectobacterium*, so that it will be named *Pectobacterium parthenii* (STARR) comb. n.

Thereby the possibility of establishing the two closely related types of bacteria from *Chrysanthemum* and *Dianthus* as varieties of this species is opened up.

Whether the *Chrysanthemum* and *Dianthus* isolates should be considered identical with *P. parthenii*, or whether the *Dianthus* isolate should be assumed to be identical with the *Chrysanthemum* isolate and both should be designated as the same variety of *P. parthenii*, or each should be designated as a separate variety of *P. parthenii*, is a matter of opinion.

In the circumstances prevailing, the differences (cf. Table XIII, p. 124) found have, however, proved reproducible; and since it will be desirable from the point of view of plant pathology to be able to diagnose with fairly great certainty the different types of isolate, the author would suggest setting up the two types of isolate from *Chrysanthemum* and *Dianthus* as two different varieties of *Pectobacterium parthenii*. The name of the *Chrysanthemum* pathogen: *Erwinia chrysanthemi* BURKHOLDER et al. (1953, p. 526) would then have to be changed to *Pectobacterium parthenii* var. *chrysanthemi* (BURKHOLDER et al.) comb. n.; and for the *Dianthus* isolate the name: *Pectobacterium parthenii* var. *dianthicola* var. n. is proposed.

Diagnosis: *Pectobacterium parthenii* var. *dianthicola* deviates from *Pectobacterium parthenii* (STARR) comb. n. as it ferments inulin, the methyl red test is positive after 2 days, negative after 6 days, the Voges-Proskauer reaction is constantly positive, and the maximum temperature for growth on nutrient agar is 35°C.

For the stirps *carotovora*, the nomenclature is clear. In 1945, WALDEE has designated *Pectobacterium carotovorum* (JONES) WALDEE as the type species for the genus *Pectobacterium*, and the name of this species thus remains unchanged (cf. JONES 1901, WALDEE 1945).

Erwinia atroseptica (VAN HALL) JENNISON has been set up by HELLMERS & DOWSON (1953) as a variety of *Erwinia carotovora*, and this view seems to be borne out by cultivation tests which now have been carried out. As a natural consequence of the foregoing, the author agrees with DOWSON, who in his new edition of "Plant diseases due to bacteria" (1957) has referred the bacterium to the genus *Pectobacterium*, and proposed the name: *Pectobacterium carotovorum* var. *atrosepticum* (VAN HALL) DOWSON (cf. VAN HALL 1902).

In accordance with the results of the abovenamed cultivation tests and the subsequent reasoning, the author has collected in the following

key the most important differentiating characters of the five different *Pectobacterium* isolates, whereby determination of these bacteria is made comparatively easy.

Key

to differentiation of "*carotovorum*", "*atrosepticum*", "*chrysanthemi*", "*parthenii*", and "*dianthicola*" isolates.
(Difco media. Indicator: bromthymol blue).

I. Colonies: on Endo agar deep red and the medium turns deep red after 5–6 days; on desoxycholate agar red; on E.M.B. agar deep red but not mucoid ("look dry"). Lactose fermented after 1–2 days. In Na-malonate no change in reaction or slightly acid. Methyl-red test constantly positive. No growth on nutrient agar at 40°C.

A. 5% ethanol in nutrient broth fermented with production of acid
.... *P. carotovorum*

B. 5% ethanol in nutrient broth not fermented or alkali produced
.... *P. carotovorum* var. *atrosepticum*

II. Colonies: on Endo agar pink and no change in the colour of the medium; on desoxycholate agar slightly pink or yellowish; on E.M.B. agar pink and mucoid, and "violet spots" seen in the colonies. Lactose fermented after 9–20 days. In Na-malonate alkali produced after 3–6 days.

A. Good growth on nutrient agar at 40°C.

1. Inulin fermented with production of acid after 1–2 days. Alkali not produced in Na-tartrate. Methyl-red test at first positive, after 6 days negative:
.... *P. parthenii* var. *chrysanthemi*

2. Inulin not fermented after 20 days. Alkali produced in Na-tartrate. Methyl-red test constantly positive:
.... *P. parthenii*

B. No growth on nutrient agar at 40°C. Inulin fermented with production of acid after 6–9 days. Alkali produced in Na-tartrate. Methyl-red test at first positive, after 6 days negative:
.... *P. parthenii* var. *dianthicola*

In the investigation and reasoning reported in the above, the author had attempted, partly to classify purely systematically the bacterium isolated from carnations, and partly to contribute towards a clarification of the very difficult taxonomic questions within the genus *Pectobacterium* Waldee. The proposal set out will, however, undoubtedly have to be considered as temporary until all the *Pectobacterium* isolates hitherto known have been examined very critically all at a time, partly on the basis of comprehensive cultivation tests both with old and young isolates, and partly by cross-inoculations with fresh isolates on many different host plants; finally, it will no doubt also be necessary to supplement

the investigations with serological analyses. In this way, a more certain differentiation of genera, species, varieties, and strains within the coliform plant pathogenic bacteria may perhaps be arrived at.

7. Dissemination of the Disease

As already mentioned, bacterial stunt of carnation is a typical vascular disease, where the bacteria are strictly confined to the vascular tissues in the stems, and as these never crack so deeply that the vessels are exposed, there should be no chance of transmitting the disease from one carnation stem to another. Even if in one way or other the bacteria could get out of the stems (through cuts or the like), there seems to be no possibility of transmitting the infection to unwounded stems. This is evidently the case in practice and it was also confirmed by the author's infection tests, which showed that a thick layer of bacterial slime on unwounded stems did not lead to any infection. It may, however, be taken for granted that the bacteria are transferred from one root system to another; practical experience and infection tests have proved this abundantly. This is possible because the individual root branches having direct connection with infected vessels become soft-rotted and are eventually decomposed, thus allowing the bacteria to ooze out into the water in the soil. Hence, the bacteria are transferred to wounded roots, invading the neighbouring plants through these channels ("secondary infection").

In the secondarily and extensively infected carnation plants the bacteria will rarely penetrate beyond some 20–40 cm up the stems, but then so many vessels may be infected that thê plants will wilt comparatively quickly, leaving the upper parts—and the cuttings there—with vessels not yet infected. The risk of taking infected cuttings from these plants just before they wilt is therefore but slight. In many cases the infection leads to complete wilting of the carnations, but some plants—both young and old and whether primarily or secondarily infected—may be so slightly attacked (i. e. have few infected vessels) that they will not react with directly visible signs of disease. Nevertheless, the bacteria are found in some of the vessels, whence they make their way out into some of the fresh shoots which are used as cuttings. It will therefore be possible to take both healthy and diseased cuttings from the same, slightly infected but healthy-looking carnation plant.

Cuttings with few vessels attacked by bacteria will not present symptoms of disease either, and will therefore be mixed with healthy cuttings. If

the cuttings are placed in water, a solution of rooting stimulant, or in a wet sand bed, the bacteria will ooze out from the bacteria-filled vessels into the surrounding water, with which they are taken up into few or many of the other cuttings, which are thus inoculated more or less extensively ("primary infection").

The author's infection tests, as well as practical experience, have shown that the bacteria from infected crops and cuttings are left in soil, sand, wooden boards, etc. and remain infectious for at least two months (cf. Section 5B, p. 113). When the roots from healthy carnations touch these contaminated materials the disease may also be readily adopted; in this way the bacteriosis is often quickly spread from plant to plant and even from one crop to another.

8. Control

A. Direct Control Measures

The control of bacterial stunt of carnation can be effected, largely, on the same lines as laid down for bacterial stem-crack of carnation. This means, for one thing, that no actual direct control of the disease in the cuttings is possible, but that "broth testing" of the cuttings in connection with soil sterilization seems to be the most effective control measures.

The bacterium is as sensitive to 8-hydroxy-quinoline sulphate as is *Pseudomonas caryophylli* but less sensitive than this latter bacterium to Agri-mycin 100. This is in good agreement with LELLIOTT's observations: that streptomycin (which is the principal constituent of the active substances in Agri-mycin 100) has comparatively slight effect on *Pectobacterium parthenii* var. *dianthicola*. On the other hand, LELLIOTT has found that aureomycin, chloromycetin, and terramycin have a strong antibiotic effect on the new carnation bacterium. Bearing in mind the negative result of the attempts at controlling bacterial stem-crack by treating the cuttings with different bactericides (8-hydroxy-quinoline sulphate, "Aretan", streptomycin, terramycin and Agri-mycin 100) (cf. Chapter I, 7A, pp. 47–70), the author has carried out only a few, orientating tests with direct control of bacterial stunt of carnation in cuttings; but these tests have confirmed that control of the bacteria in the vessels is extremely difficult, if at all possible. The tests were carried out so that 10 diseased cuttings of the variety "Dark Red Sim" were placed in a 1-litre beaker containing 200 ml 0.5$^{0}/_{00}$ 8-hydroxy-quinoline sulphate, while 10 other diseased cuttings of the same variety

were put in a similar beaker with a 200 ml 5.6$^0/_{00}$ Agri-mycin 100 solution for 2 days at ordinary room temperature. Thereafter, discs of stem were taken from the treated cuttings as described (p. 34) and laid on PDA in Petri dishes; but, apart from the lowermost disc, colonies of *P. parthenii* var. *dianthicola* developed at most of the discs. Further attempts at direct control of the bacteria in the stems were therefore given up.

Consequently, the author is of the opinion that control of bacterial stunt of carnation with the use of bactericides is applicable only to disinfection of dishes and utensils by washing with 1$^0/_{00}$ 8-hydroxy-quinoline sulphate or other strong disinfectants. Furthermore, as in the case of bacterial stem-crack, it is recommended to avoid the use of solutions of rooting stimulants, which should be replaced with powder treatment, and also to take care that benches and media for cuttings are renewed or carefully steamed (at least for one hour) after termination of each stock of plants.

Bacterial stunt of carnation seems, incidentally, in a higher degree than bacterial stem-crack to spread in older stocks of carnations, and this had led to some investigations of the chances of checking the attack by watering with a 2$^0/_{00}$ aqueous solution of 8-hydroxy-quinoline sulphate. The waterings were carried out in a couple of nearly 2-year-old stocks of the varieties "Ember Sim" and "Dark Red Sim", and so that, round the first row of apparently healthy plants surrounding a so-called "gap" with plants attacked by bacteria, an about 15 cm deep trench was scraped, into which so much "8-hydroxy-quinoline water" was poured that the trench was filled completely with the liquid a couple of times. Pouring was done a couple of times during the first week and later repeated 4 times at intervals of 1 week. Even if observation for a longer time would be required for the proper assessment of the effect of this treatment, it looks already now, after only 6 months have passed, as if the disease has been inhibited, and the carnations do not appear to be harmed by this treatment.

B. Indirect Control Measures

Although the bacterium cannot resist 4 months storage in sterile garden soil, the organism does not always die out in infested carnation beds after so short a time. In practice the soil seems to remain infectious for so varying periods that at the time being it is impossible to say when the bacterium definitely dies out in a certain soil.

The most effective means of controlling bacterial stunt of carnation is undoubtedly the biological testing of the sterility of the interior tissues of the cuttings. For bacterial stem-crack of carnation, testing of the cuttings is effected according to the so-called "broth method", which has been elaborated by the author, and which in the barely 2 years the method has been used has proved a very effective "weapon" against the disease.

As mentioned already in the introduction, one of the first attacks of bacterial stunt of carnation discovered in Denmark was found among "nutrient broth tested" cuttings of the variety "Lucky Strike", and later the disease has been ascertained in other "nutrient broth tested" carnations, for example in a lot of "Cardinal Sim" that had been exported to Sweden, from where the diseased plants were returned for investigation by the author.

There can thus be no doubt that it was impossible to "detect" by the "nutrient broth testing", as performed up to the spring of 1956, all the cuttings attacked by bacteria in a given lot of cuttings. In the light of the good results obtained with the "nutrient broth testing" when applied to bacterial stem-crack, the sudden failure of this method was not immediately understood.

The individual links of the "broth method" were therefore once again made the subject of careful investigation, and it was then established that the surface sterilization of the carnation stems was not so intense that the bacteria inside the vessels were killed; even after further intensification of both the washing-off and flaming of the carnation stems, *Pectobacterium* colonies could easily be isolated from the diseased vessels. An examination of the medium also showed that the bacterium grew particularly well in the glucose-containing ($1^0/_0$) broth used.

There would thus appear to be nothing to prevent the bacteria from the attacked vessels in the stem-pieces from growing out into the medium, numerous random inspections showed that in many cases the bacteria could easily be demonstrated in the nutrient broth. On the other hand, the diseased plants among the tested cuttings proved that not all attacks were detected during the testing. The reason for this was assumed to be two-fold: partly that "*dianthicola*" is found very sporadically in the carnation stems, so that it would be possible in certain cases to cut out from the cuttings stem-pieces which are free of bacteria even though other portions of the vessels contained bacteria; and partly that the temperature in the incubators, which, regarding the high optimum

temperature for *Pseudomonas caryophylli*, was kept at 32–33°C, was too high for "*dianthicola*".

This latter problem was solved very quickly, as cultivation tests with the "*dianthicola*" isolates showed a considerable inhibition in the growth on PDA at temperatures above 29–30°C. So there was no longer any doubt that the high temperature in the incubators has been an essential obstacle to the growth of "*dianthicola*",—for which the optimum temperature is 25–27°C—and this must undoubtedly be considered the main cause of the failure of the bacteria in the stems from the slightly infected plants to make the media turbid while incubated. Therefore the cuttings concerned could not be "labelled" diseased and be discarded.

The temperature in the incubators was then lowered to 25–27°C, and at the same time the duration of the stay of the media in the incubator was extended to 6 days[1]) to further allow the bacteria in the very slightly infected pieces of stem to grow out into the medium.

To throw light on the hypothesis about the uneven destribution of bacteria in the vessels, some tests were performed, with isolations partly from naturally infected cuttings and partly from cuttings which for 2 days had been taking up an aqueous suspension of isolates Nos. 98 and 99 in a mixture. The isolations were carried out so that 5 cuttings from each set were cut up into quite thin slices (about 1–2 mm thick) which were placed in numerical order on PDA in Petri dishes. Each slice was made with a sterile razor blade to prevent transfer of bacteria from one cut to the next; the dishes with the many slices were incubated at 27°C. After 5 days, bacterial colonies had formed at all slices of stem from the naturally infected cuttings, but in the dishes with slices from the artificially infected cuttings it happened in a few cases that a single slice remained sterile, although the slices cut out above it were infected by bacteria; incidentally, this fact was also noticed during the "absorption tests" with *Serratia marcescens* (p. 34) carried out in conjunction with the examination of bacterial stem-crack of carnation.

As such an uneven distribution of the bacteria in the stems has not been observed by numerous isolations of both heavily and slightly, naturally infected cuttings, it was assumed to be a phenomenon particular to the inoculation method applied. However, it is possible that the same uneven distribution of the bacteria nevertheless may occur in a few

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[1]) After a period of 6–8–10 days incubation it has never been possible to demonstrate anything but saprophytes in the broth tubes.

very feebly infected cuttings. In order to avoid taking out bacterium-free pieces of stem from slightly infected cuttings, it was resolved that each of the 3–4 stem-pieces cut off during the nutrient broth testing should in future be well over 0.5 cm long, so that the tested piece of stem would be 1.5–2.0 cm long in all and the chance of not having any bacteria transferred to the medium would be diminished.

The testing method thus modified was used in two nurseries for 18 months, during which period more than 200,000 cuttings were tested, but up to May 1957 it was not possible to demonstrate diseased plants among the carnations "approved" by the testing, although numerous random samples were taken.

However, in May 1957 in one of the nurseries about half (55% and 53%, respectively) of two lots of young, tested plants of the varieties "Scania" and "Galasso" (both "Sim" varieties) were found to be attacked by "bacterial stunt of carnation", *Pectobacterium parthenii* var. *dianthicola* being isolated from the diseased plants.

A closer investigation of the "history" of the two lots of diseased carnations revealed no actual faults in the testing, but it was ascertained that the quite young, newly potted plants in certain periods had been left for several months on benches with moist *Sphagnum*, on which also diseased plants had previously been standing. It was therefore possible that the tested plants had become infected from the *Sphagnum*. On the other hand, the author was still of the opinion that, after all, a few diseased cuttings might somehow have passed the testing inspection and communicated the bacteria to the healthy cuttings already in the rooting period. Such a "slip" in the inspection could be supposed to take place by a simple mistake during testing or if, as previously mentioned, the bacteria in but slightly infected cuttings occur so sporadically in the vessels that—despite a rather considerable lenght—the stem-pieces cut off during testing are free from bacteria, while the other parts of stem of the cutting contain a few pathogenic bacteria. The author of this paper has previously attempted to prove by experiments (p. 135) and numerous random tests the truth of this hypothesis, but so far it has been impossible to demonstrate any heterogeneous distribution of bacteria in the vascular tissue of carnations; nor have any diseased plants been observed among the tested cuttings. Since the possibility of diseased cuttings having passed the inspection could not be completely ruled out in this way, however, it was decided to use both lots of carnations (749 "Scania" and 154 "Galasso") for a very thorough investigation for the presence of bacteria in the stems, in the hope of throwing

light on the distribution of the bacteria in naturally infected cuttings.

For this investigation, 500 cuttings of the variety "Scania" were taken from 412 healthy-looking, though infected[1]), plants and 200 cuttings taken from both healthy and diseased[1]) though healthy-looking individuals (about half of each) of altogether 154 plants of the variety "Galasso". The total of 700 cuttings were then subjected to a special testing, the three lowermost internodes of each cutting being tested separately. Each cutting was tested three times at different levels of the stem (one testing for each internode); and as all turbid media were spread on PDA in Petri dishes, it should be possible to prove in this way whether certain stem parts were sterile while other parts of the same stem contained bacteria.

The results of the investigations fully confirmed the author's previous observations: that it is sometimes possible to take perfectly healthy cuttings from diseased plants (namely, the cuttings having no connection with the infected vascular tissue), as no less than 11 (= 2.2$^0/_0$) of the 500 cuttings which had been taken from diseased mother plants exclusively were found to be free from bacteria in all the three internodes. 17 cuttings (3.4$^0/_0$) also appeared to be healthy, but in one or more of the testings contaminations occurred (ascertained by streaking on PDA). Althogether 11 + 17 = 28 (= 5.6$^0/_0$) were thus found to be perfectly healthy. Of the rest of the cuttings, 466 (= 93.2$^0/_0$) were found to contain bacteria through all three testings, whereas 6 (= 1.2$^0/_0$) of the cuttings were found to be infected only in one or two of the three internodes.

Of the 200 cuttings picked from diseased and healthy plants, altogether 76 (= 38.0$^0/_0$) cuttings were found to be entirely free from bacteria in all internodes; 45 (= 22.5$^0/_0$) were likewise healthy but contaminations were found in one or several of the media. In all, 121 (= 60.5$^0/_0$) cuttings were healthy. Among the rest of the cuttings, 62 (= 31.0$^0/_0$) were found to be infected through three testings whereas 17 (= 8.5$^0/_0$) showed a content of bacteria in only one or two of the three internodes.

By further examination of the media from the abovenamed altogether 6 + 17 = 23 cuttings (the cuttings in which the three testings were different) it was proved for the first time that the bacteria in the vessels of the stems of naturally infected carnation cuttings may be distributed so sporadically that the lowermost internode of 4 (= 0.6$^0/_0$) of the cuttings

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[1]) The infected plants were selected by examining transverse sections taken from the base of the stem and finding distinctly discoloured vascular tissues, from which *Pectobacterium parthenii* var. *dianthicola* was isolated.

was found to be sterile, while the upper stem parts from the same cuttings contained pathogenic bacteria. In 5 ($= 0.7^{0}/_{0}$) of the cuttings, only the centre internode was found to be infected, whereas in these cuttings both the lowermost and the uppermost internodes were perfectly free from bacteria. Finally, only the lowermost or the two lowermost internodes in altogether 14 ($=2.0^{0}/_{0}$) cuttings were found to contain pathogenic bacteria, but these "cases" would of course be found under the normal testing. However, the cuttings (here 0.6 and $0.7^{0}/_{0}$, respectively), where only the uppermost or the centre internodes are infected, while the lowermost internode (on which the testing is made) is healthy are the cuttings which now and then slip through the testing inspection.

The examination of the cuttings from the varieties "Scania" and "Galasso" thus showed that the high percentage of disease in the tested carnations was due not only to infection during the stay of the plants on infested *Sphagnum*, but it could be assumed also that a few infected cuttings passed the tests—on account of the uneven distribution of the bacteria in the vessels—and actually contained bacteria and so spread the disease on the propagation bench.

It must then be admitted that, although it is possible, by means of the above described "broth method", to sort out from but slightly infected lots of cuttings so many diseased individuals that it is generally impossible to find the few diseased plants, which undoubtedly occur among the tested carnations, it may happen in severely infected lots that no less than $0.6–1.3^{0}/_{0}$ diseased cuttings pass the inspection. The slightly infected cuttings which are not sorted out by the tests—the so-called "overrunners"—therefore go with the healthy ones and may infect many neighbouring cuttings unless special precautions are taken to prevent it.

Such spreading of the disease in the propagation bench can, however, be prevented by setting the tested cuttings separately in sand, each in a small flowerpot (5 cm) of its own, and suspending the pot in chicken wire netting so that it will not touch the bottom of the bench, or placing it on chicken wire netting supported by a 3–5 cm thick layer of shingle (1–3 cm) (Fig. 28) on an ordinary propagation bench (both sand, pots, shingle and bench materials must be sterile); if the young, rooted plants are also kept in pots on a well-drained stone (shingle) base and continually repotted before the roots grow through the bottoms of the pots, it should be possible to keep the carnations for 3–4 months without becoming infected by neighbouring plants.

Fig. 28. Carnation cuttings inserted in "Peralite" – a new medium (of vulcanic origin) for cuttings – in pots (5 cm), placed on chicken wire netting supported by shingle on the bench.

Incidentally, experience of bacterial stunt of carnation shows that after about 3 months the bacteriosis in the slightly infected carnations has generally developed to such an extent that it can easily be seen, by transversely cutting the root collar, which plants are diseased, as they have brown or water-soaked vascular tissues. After the said period it will therefore be possible, by cutting the plants, to sort out directly most of the infected plants. However, the author has often proved that even the most careful inspection, with the aid of a hand lens, cannot point out the very slightly infected carnations (some few per cent. slip through). Therefore the cuttings from the remaining (healthy looking) plants will have to be tested according to the "broth method". Further, if the plants after the second testing are found with no signs of discoloration in the stems (cross sections of stem are made again), the third lot of tested cuttings must also be tested but can be rooted in the usual way. If, however, infected plants are found among the second time tested carnations, 2–3 more testings and separate propagations will be necessary.

The uneven distribution of bacteria in the vessels of carnation stems may not at all occur in plants tested in the period October–February, the normal time for propagation of carnations in Denmark; at least, infected plants have never been found among winter propagated tested lots. The author therefore supposes that this is explained by the fact that during the said period the low temperatures are retarding the growth and the movements of the bacteria as well as the growth of the plants, and so is also the sap current retarded to some extend. Therefore, perhaps, the testing is more reliable in winter time, and that may be the reason why it is so difficult – if at all – to find infected plants among tested carnations propagated in the normal period.

The propagation technique described here has been carried out in a single Copenhagen nursery for altogether 5000 cuttings in the summer of 1957, and it has thereby been established, that the setting technique proper can very well be carried out. The author therefore considers it highly probable that by this combined testing and propagation technique it is possible in the course of only 6 to 18 months to provide plants which can with certainty be considered healthy mother plants. Cuttings taken from these mother plants can then be rooted and planted in the usual way.

Chapter III

The Incidence of the Carnation Bacterioses -

as well as of *Phialophora cinerescens*, *Fusarium dianthi*, and *Fusarium culmorum* – in Denmark

1. Introduction

When, in the spring of 1956, the investigations of the carnation bacterioses reported on in the foregoing had advanced so far that the author felt confident of being able to distinguish the two bacterial diseases from each other with certainty, a comprehensive investigation of stocks of Danish carnations was carried out with the object of finding out how far these two diseases had spread in Denmark. During this investigation it was revealed that the Danish perpetual-flowering carnations were also extensively attacked by fungal diseases caused by *Phialophora cinerescens* (WOLLENWEBER) VAN BEYMA, *Fusarium dianthi* PRILL. & DEL., and *Fusarium culmorum* (W. G. SM.) SACC., respectively, the firstmentioned of which had not previously been observed in Denmark.

The abovenamed fungal diseases are so inseparately bound up with the results of the investigation and the question of the causes of the "wilt disease of carnation" in general that a brief mention will be made of them, partly in this Chapter and partly in Chapters IV (p. 150) and V (p. 167).

2. Material and Methods

A. The Plant Material for the Investigation

The investigation was arranged in the way that from carnation nurseries all over the country 3–5 wilting plants were taken from each clone[1]) in which "wilt disease"[2]) was found (Fig. 36, p. 156). With a few

[1]) The word clone is used here to denote the stock of the same age which in practice is termed a "lot" or "culture" of plants, and obtained by cuttings under the same variety name, irrespective of the origin of the mother plants.

[2]) "Wilt disease" means here any form of wilting of carnations; however, in the investigation, only plants that were about to wilt (dying), not entirely dead plants, were taken out.

exceptions, all the plants were selected by the author himself; and, after having been dug up with its root, each plant was immediately put in a plastic bag (Fig. 29) and examined in the laboratory on the same day, or within 2 days at the latest.

B. Investigation Technique

Judgment of the plants taken out. The investigation was carried out so that the colour of the plant and the condition of the root system were judged first, it being ascertained whether the roots were healthy or had half rotted away or were rotted completely. Next, the root and about 5 cm of the stem were cut off, and the cross section of stem thus produced was examined macroscopically (cf. Fig. 39, p. 168) as well as microscopically. Thereby the presence of bacteria or/and fungal hyphae was discovered in the vessels, and then the run and height of the brown vascular tissue in the stems of the plant were ascertained by observing cross section of stem through a hand lens for each 10 cm up the stems.

Isolation technique. From the basal portions of the stems as well as from the distal parts with discoloured vascular tissue, attempts were made at isolating micro-organisms. These isolation tests were carried out so that the stem-piece concerned—as a rule between 5 and 10 cm long—was first washed thoroughly in "oxyspirit", which was then burnt off by slight flaming over a Bunsen burner; finally, discs of stem of about 2–5 mm thickness were cut off with sterile razor blades, and some of these discs were placed directly on PDA in Petri dishes (Figs. 30, 31, and 32), while others were put in a few millilitres of sterile, distilled water in test tubes, from which a few drops of the water (suspension) were streaked on to PDA in Petri dishes (10×1 cm) (Fig. 24, p. 112) after about 4–6 hours' standing.

From each plant, 4–10 (15) cross sections of stem were cut, both from the basal and the distal portions of the stem, but in several cases—particularly where the symptoms were not perfectly distinct—many more isolations were made from each plant, so that as a rule 25–60 isolations, distributed with 5–10 discs of stem in each Petri dish (Fig. 32), were made from each clone from each nursery. All the dishes were left on tables in an ordinary laboratory at about 18–22°C, and after 3–4 days each dish was examined partly through a hand lens and partly through a microscope.

Fig. 29. Three carnation plants (Journal No. 16) in plastic bag, such as the samples of wilting carnation plants were taken. ×0.1.

3. Results of the Investigation

The investigation of the stocks of Danish carnations comprised altogether 53 nurseries, which means that, practically speaking, all Danish carnation growers have been involved in the analysis.

Among the 53 nurseries there were but two whose stocks of plants displayed no sign of "wilt disease" of any kind and therefore, for the present, had to be declared healthy. Three nurseries had given up the growing of carnations on account of the "wilt disease", which gradually had destroyed the plants completely. Another nursery had stopped growing carnations for some other reason and one did not want to participate in the investigation. The original number was thereby reduced by 7 so that, actually, the investigation comprised only 46 nurseries, from which altogether 147 clones (cultures or crops), distributed in 31 varieties, were examined. A review of the names of the varieties investigated and the diseases of which they were found to be infected, as well as the distribution of the various diseases on the different clones and varieties, is given in Table XV, p. 147.

The result of the investigation was surprising in several respects. In the first place, "bacterial stem-crack of carnation" (Fig. 3, p. 19) was seen only once; secondly, "bacterial stunt of carnation" (Fig. 20, p. 101) was found spread extensively in $26.5\,^0/_0$ of the clones investigated; and, thirdly, it was found that $54.4\,^0/_0$ of the clones investigated were attacked by a fungus—*Phialophora cinerescens*—(Fig. 32, p. 148). Finally, it was not expected that "*Fusarium*" (*Fusarium dianthi* and *Fusarium culmorum*) would be demonstrated in only $10.2\,^0/_0$ of the plants (Fig. 31, p. 146). In $8.2\,^0/_0$ of the plants it was impossible to demonstrate any pathogen at all; in these cases the discs of stem stayed sterile, and the cause of the disease was presumed to be of a non-parasitic nature.

Further mention of the results of the analysis is made in the following.

A. Cause of the Disease: Bacteriosis

As may be seen from Table XV, bacterial stem-crack of carnation (*Pseudomonas caryophylli*) was observed in a single variety ("Laddie Sim") only, a clone which quite recently had been imported into Denmark direct from the U.S.A.

Otherwise, bacterial stem-crack of carnation seemed to be entirely eradicated from the stocks of Danish carnations. The reason for this is, no doubt, primarily that very rigorous control measures were taken in

Fig. 30. *Pectobacterium parthenii* var. *dianthicola*. Photographed 3 days after the discs of stem from "White Sim" had been placed on PDA. Note the lobate margins of the colonies (already after 3 days). Photographed 15.6.1956. ×1.1.

the nursery where this disease was first observed and that at the same time testing of cuttings for mother-plant stocks and for sale was started.—Nevertheless, it was remarkable that the disease could not be traced in any other nursery, in spite of the fact that several of the diseased cuttings had been distributed to a number of Danish nurseries before the cause of the disease was originally established with certainty and the sale of the attacked varieties stopped. There are probably several causes of the disappearance of the bacteriosis from these nurseries. Firstly, the infected plants that were sent out in 1951–53 were generally so severely diseased (25–75 $^0/_0$ diseased was not unusual) that most of the plants died very quickly, for which reason the growers had all the lots which were infected with disease destroyed completely. Secondly, it is common practice in this country to either steam the soil on the termination of each crop of carnations or to replace the soil with fresh field soil, and there can hardly be any doubt that the sterilization or renewal of the soil, as well as the washing of the benches after removal of the diseased carnations, have been done very thoroughly, so that the bacteria have had but a slight chance of surviving. As, furthermore, the bacterium has such a high optimum temperature that it must be assumed to be difficult for it to grow in our cool climate (soil), at least most of the year, and it, moreover, makes certain demands in respect of the nature of the "medium", it may not be so strange, after all, that bacterial stem-crack of

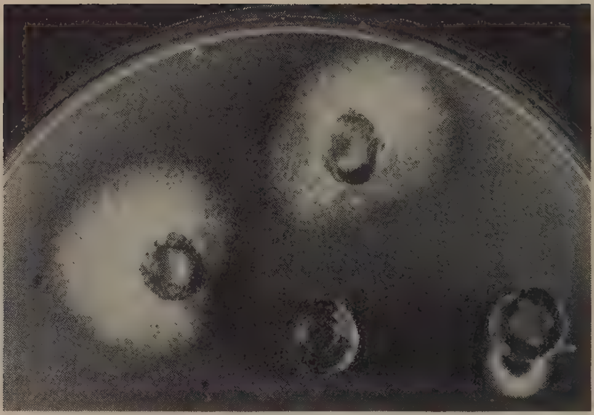

Fig. 31. *Fusarium dianthi* PRILL. & DEL. Photographed 3 days after placing of discs of stem from "Shirley Ann" on PDA. Compare the abundant mycelial growth (the two upper (big) mycelia) with the very slight growth of *Phialophora* in the same period (Fig. 32). ×1.1.

carnation may now be considered, practically speaking, to have been eradicated in Denmark.

During the pathological analysis of the stocks of Danish carnations it was, moreover, ascertained that bacterial stunt of carnation (*Pectobacterium parthenii* var. *dianthicola*) (Fig. 30) was more widespread than generally supposed, no less than 26.5 $^0/_0$ of the stocks—or 39 clones distributed in 10 varieties—being found to be attacked by this new disease (Table XV).

Bacterial stunt of carnation has been spread all over the country some years before the existence of the disease was discovered at all, but, because of its comparatively low temperature requirements, the causal bacterium of this disease seems to be better adapted to the Danish climate (this bacterium is closely related with the bacterium which produces "black leg" in potatoes and which is found widely spread in this country) than is the pathogen of bacterial stem-crack of carnation. And since the bacterium, besides, grows well as a saprophyte, it is undoubtedly a good deal more difficult to control, and is thus also more dangerous, than the pathogen of bacterial stem-crack of carnation.

Table XV. Review of varieties of carnations, numbers of clones, and the diseases (bacterioses and mycoses) which a pathological analysis made in Denmark in June–July 1956 proved them to be attacked by.

Variety of carnation	Colour	No. of clones attacked by:				
		Bacteriosis		Mycosis		Non-parasitic factors
		Pseudomonas	*Pectobacterium*	*Phialophora*	*Fusarium*	Sterile stem discs
White Briary	White			1		
White Sim	–		7	14	2	2
Geiger Sim	White & red			1		
Asa Thor	Yellow			2		
Harvest Moon	–		5	4		
Hoosier Gold	–					1
Maine Sunshine	–					1
Midas	–			1		
Shirley Ann	–			1		1
Skyliner	–				1	
Apollo	Pink		5	2		
Edw. Winkler	–				1	
Elisabeth	–				1	
Eros	–					1
Hermosa	–			1		
København	–			3		
Mina Brenner	–			2		1
Petersen's Pink Sim	–		4	21	5	2
Sidney Littlefield	–		2	3	1	
Laddie Sim	Rose	1				
Salmon Sim	–		8	3	1	
Betty Lou	Medium red			1		
Ward	–			1		
Cardinal Sim	Red		2	1	1	
Dark Red Sim	–		4	4		
Ember Sim	–		1			
Majestic (Sim)	–				1	
Scania	–			1		
Tetra Red	–			1		
William Sim	–		1	11	1	3
Tetra Sahra Jane	Lilac			1		
Altogether 31 varieties and 147 clones		1 (0.7%)	39 (26.5%)	80 (54.4%)	15 (10.2%)	12 (8.2%)

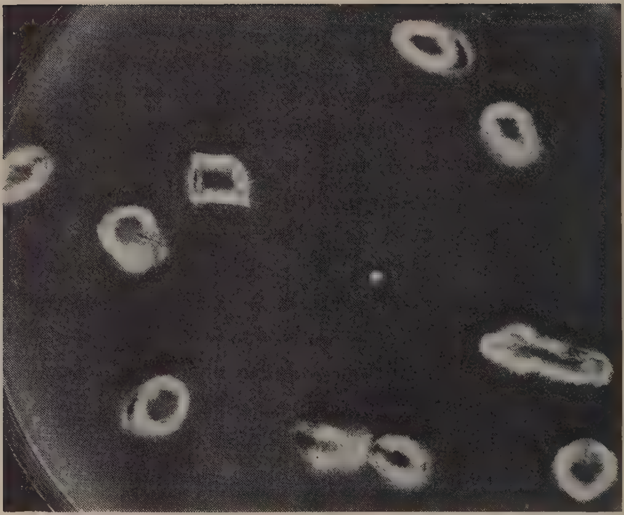

Fig. 32. *Phialophora cinerescens* (Wollenweber) van Beyma. Photographed 3 days after placing of discs of stem from "Petersen's Pink Sim" on PDA. Note the mycelial growth from the xylem; see also Fig. 33. ×1.1.

B. Cause of the Disease: Mycosis

The pathological analysis of the stocks of Danish carnations, revealed that no less than 54.4% of the stocks—or 80 clones distributed in 22 varieties—were attacked by a fungus, a Hyphomycete which was determined as *Phialophora cinerescens* (Wollenweber) van Beyma (cf. Table XV and Figs. 32, 33, 34, and 35)—a carnation pathogen which is much dreaded in England and Holland but the existence of which in this country has been the subject of discussion for many years.

Now there can, however, no longer be any doubt that this serious carnation disease is found in Denmark and is even wide-spread. Nor can there be any doubt that this fungus is the most frequent cause of the so-called "wilt disease" or "svindsot". The demonstration of this fungus may thus be taken to present the solution of the enigma—or at least an essential part—of the cause of "wilt disease" which has been harassing our perpetual-flowering carnations for many years (a more detailed mention of the disease proper is given on p. 150).

Wilting of perpetual-flowering carnations caused by the *Fusarium* species, *Fusarium dianthi* Prill. & Del. and *Fusarium culmorum* (W. G.

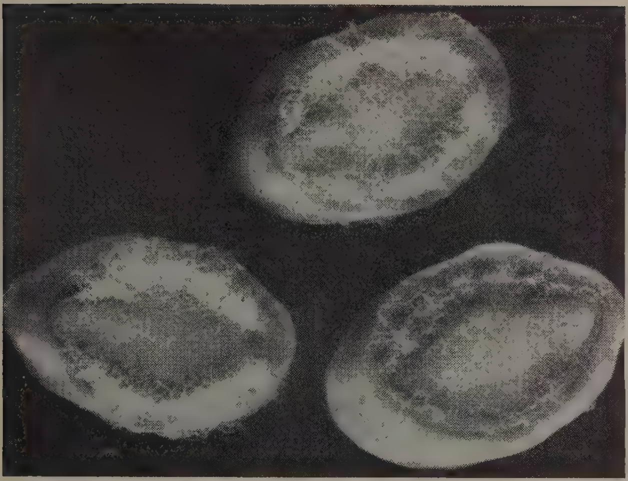

Fig. 33. Similar to Fig. 32; parts of Fig. 32 enlarged 4 times.

Sm.) Sacc. (Fig. 31) has formerly been demonstrated in this country (The Danish Plant Pathological Research Station, 1922, 1932). In the author's investigations this was found in 10.2⁰/₀ of the clones—or 15 clones distributed in 10 varieties (Table XV). These figures are presumably lower than would generally be expected, but this is probably due mainly to the fact that a large number of the "*Phialophora* cases" and perhaps also some of the "bacteria cases" have erroneously been believed to be attacks of *Fusarium* spp. (a more detailed mention of the disease is given on p. 162).

C. Cause of the Disease: Non-Parasitic Factors

In conclusion, it may be mentioned that in 8.2⁰/₀ of the samples—or 12 clones distributed in 8 varieties (Table XV)—it was impossible to isolate any pathogen from the wilting plants. What the cause of the disease has been in these cases was not further investigated, but in most cases it was likely that the roots of the plants had been damaged by adverse cultivation measures (excessive watering, too deep planting, frost, etc.) and so these cases were diagnosed as non-parasitic diseases.

Chapter IV

APPENDIX:

Wilt Disease in Danish Perpetual-Flowering Carnations, Caused by Parasitic Fungi

1. Fan-Mould of Carnation

(Phialophora cinerescens (WOLLENWEBER) VAN BEYMA*)*

A. Introduction

As mentioned in Chapter III, it was found during the pathological analysis of the Danish perpetual-flowering carnations that 54.4$^0/_0$ of the clones (crops) investigated—or 80 clones out of 147—were attacked by the fungus *Phialophora cinerescens* (WOLLENWEBER) VAN BEYMA, a fungus not previously demonstrated in Denmark but known in England, Holland, Germany and the U.S.A.

Phialophora cinerescens was originally described by H. W. WOLLENWEBER (1929) as a new *Verticillium* species, and because of its greyish appearance in culture it was given the species name of *cinerescens*. According to WOLLENWEBER's diagnosis, this fungus has cylindrical-ellipsoidal, at first hyaline, later greyish conidia measuring 4.4 × 1.75 μ, on an average, or varying between 3–7 × 1.5–3 μ (most often 3.8–4.9 × 1.7–2 μ) and abstricted from the tip of conidiophores the side branches of which are as a rule placed two or three together. Obviously, WOLLENWEBER is of the opinion that the conidiophores are mostly like those of *Verticillium*, but he states that they may also be suggestive of those of other genera, e. g. *Spicaria*. From the accompanying drawing it appears plainly, however, that this fungus cannot be any *Verticillium*, and VAN BEYMA (1940) has in fact pointed out that *Verticillium cinerescens* WOLLENWEBER is no *Verticillium* but should properly be referred to the genus *Phialophora* MEDLAR (1915). The fungus thus belongs among the *Chalareae*, a tribe under *Dematiaceae*, within *Hyphomycetales* (*Fungi imperfecti*).

Fig. 34. *Phialophora cinerescens* (WOLLENWEBER) VAN BEYMA. At the bottom right-hand corner a quite young conidiophore is seen with but a few phialides; a couple of the phialides bear conidia. Further, some typical fan-shaped collections of phialides are seen, with a single conidium here and there. The preparation is stained with phloxin. See also Fig. 35. × about 300.

In this connection it should be noted that N. F. CONANT (1937) has demonstrated that the genus *Cadophora* LAGERBERG et al. (1927) is identical with *Phialophora* MEDLAR.

The fungus isolated by the author from the many carnations is not in excellent agreement with the descriptions given of the genus *Phialophora* MEDLAR, but agrees well with the descriptions by WOLLENWEBER (1929) and, later, by WICKENS (1935) of *Phialophora cinerescens*. It has conidiophores of a penicillium-like or candelabrum-like structure and bearing at the tip bottle-shaped conidiogonia or phialides (Fig. 34), from which cylindrical-ellipsoidal, at first hyaline and later slightly smoke-coloured conidia are abstricted singly (Fig. 35); by measuring 20 spores the following dimensions were found: 2.9–6.9 × 1.3–2.3 μ which are in excellent agreement with the descriptions given by WOLLENWEBER and WICKENS.

The author of this paper agrees with VAN BEYMA that the fungus cannot be a species of *Verticillium* as the conidiophores are built very

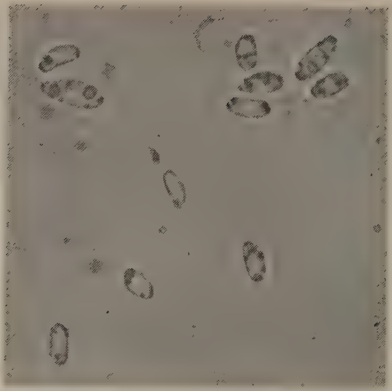

Fig. 35. Conidia of *Phialophora cinerescens* (Wollenweber) van Beyma. ×1500.

much like a *Penicillium* and as the hyphae, phialides, and conidia are in good agreement with the description of *Phialophora*. On the other hand, these conidiophores of penicillium-like build (Fig. 34), are not typical of the genus *Phialophora* either, but a single species—*Phialophora malorum* McColloch (1944)—has conidiophores which to some extent resemble those of *Ph. cinerescens*. They are, however, not identical because of the larger conidia of *Ph. malorum*.

It may be a question whether these two fungi should be incorporated in *Phialophora* Medlar at all, but as it has not been possible for the present author to find a genus in which at least *Ph. cinerescens* could be better placed, it was decided, ad interim, to retain the fungus in the genus *Phialophora*.

In Denmark "wilt disease of carnation" has been known for many years, perhaps as early as 1915–20. And it may not be amiss to presume that this wilt disease has been caused mainly by *Phialophora cinerescens*, even if this fungus has never been demonstrated and even if the disease has sometimes been diagnosed in this country as attack of *Verticillium* wilt; this is suggested by the following facts, among others:

The genus *Verticillium*, more particularly the species *V. albo-atrum*, is well-known in Denmark, where diseases caused by this fungus generally are named "Kransskimmel" (= "*Verticillium* wilt"). As the carnation disease is highly resembling attack of *Verticillium* wilt and as the disease for several years has been referred to in foreign literature (e. g. by Wickens 1935, White 1935, 1936, 1938, Brown 1938, Williams

et al. 1940, Guba 1947, 1949) as attack of *Verticillium*, and as "*Verticillium* wilt" has been observed a couple of times in Denmark (The Danish Plant Pathological Research Station 1932 and 1936) in connection with wilted perpetual-flowering carnations, it is conceivable that "wilt disease of carnation" in this country has for many years been known as "Kransskimmel" (= "*Verticillium* wilt"). It should be added, however, that, as far as the author is able to estimate, no thorough isolation tests from carnations and determination of the isolate as a *Verticillium* species have been carried out in this country so far; the few determinations available are all believed to be based on a macroscopic assessment of the pathological picture, taken in conjunction with foreign descriptions. However, the author feels confident that the estimation has been right in a certain sense and that the diseased carnations have actually been attacked by *Verticillium cinerescens* Wollenweber = *Phialophora cinerescens* (Wollenweber) van Beyma. If the determinations had been supplemented with proper isolation tests, the embarrassing confusions of *Verticillium* and *Phialophora* would undoubtedly have ceased immediately, for the two fungi are not like each other at all, neither culturally nor under the microscope.

The conidiophores of *Phialophora cinerescens* appear like widespread brushes or fans (Fig. 34), and in an attempt at introducing a new name in substitution of the misnomer "Kransskimmel" (= "*Verticillium* wilt") hitherto used, the author would therefore propose that the fungus—and the disease—should be called "fan-mould of carnation" (in Danish: "Nellikens Vifteskimmel").

B. Description of the Disease

History and distribution. Fan-mould of carnation was first observed in 1929 by H. W. Wollenweber, who failed, however, to give any detailed description of the actual disease in perpetual-flowering carnations. Such a description was later on given by G. M. Wickens (1935) in his very elaborate work on wilt, stem rot, and dieback of the perpetual-flowering carnation in England. Wickens did not occupy himself with bacterial diseases, but, then, it is doubtful whether these were in existence in England at that time.

Besides from Germany and England, fan-mould of carnation is also reported from Holland (Roodenburg 1945, Noordam 1950), U.S.A. (Guba 1947, Schneider et al. 1950), and France (Moreau 1957), and the present author has (in 1956 & 1957) isolated it from carnations from Denmark, England, Norway, and Sweden.

Symptoms and etiology. Wickens (1935) states that the *Phialophora* wilt disease manifests itself by gradual wilting of the carnations, during which the leaves fade and become straw-coloured and are often twisted somewhat. When the stems of older, attacked plants are cut transversely, a plainly annular, brown discoloration is seen right under the bark (Fig. 39, p. 168). Incidentally, it is characteristic that the fungus produces no actual rot (decomposition of tissue) of the attacked tissues of the stem.

These symptoms have also been observed in naturally infected Danish carnations and during infection tests with an isolate of *Phialophora cinerescens* isolated by the author. The inoculation was effected by placing pieces of a PDA culture close to bruises on the bases of the stems (below soil level) of 10 (2-month-old) carnation plants of the variety "Scania". In the course of 2 months the first lower leaves began wilting with a yellow-reddish colour, and after 5–6 months all the fungus inoculated carnations had wilted completely. From these, *Phialophora cinerescens* was isolated constantly, while 10 check plants, which had only been inoculated with sterile agar pieces, remained healthy.

The author of this paper can therefore fully endorse the symptom description of Wickens and can, furthermore, add that the brownish discoloration of the tissue right inside the bark is not seen so distinctly when the collar of the carnation, i. e. the lowermost 5–10 cm of the stem, is transversely cut, but it appears very distinctly when the thinner stems are cut through about 20–40 cm up the plant.

It is also characteristic of carnations attacked by "fan-mould", that, as a rule, the roots appear to be surprisingly healthy in the macroscopic examination, thus they are generally not torn off if the plant is pulled out of the soil. The top shoots and the topmost leaves of the wilting plants become irregularly reddish-mottled; a symptom which has not been noticed by Wickens, and has hitherto not been observed in connection with any of the "wilt diseases of carnation" known heretofore.

Further, Wickens mentions that the disease follows the cuttings as hyphae inside the stems—as many as 13% of diseased cuttings were found in a single lot—and that the fungus spreads partly upwards in the stems and partly downwards into the roots, resulting in wilting of the plants. The wilting may take place suddenly but may also take an insidious course so that, first, one or a few of the lowermost leaves will wither, often with brown ribs, and, next, a twig or smaller portion of the plant wilts; eventually, the whole carnation will wilt, however, assuming at first an inconspicuous greenish-grey discoloration; later on

the wilted plants become light straw-coloured. The fungus is found widely dispersed in the stems of the carnations, and it is by no means certain that the presence of hyphae in a twig will immediately give symptoms of wilting. Consequently, WICKENS also found that it is possible to take healthy cuttings as well as diseased ones from the attacked plants; in the instance reported above, where 13% diseased plants were found in a lot of cuttings, the cuttings had just been obtained from apparently healthy mother plants!

WICKENS description of the symptoms is in perfect agreement with the disease found in the Danish carnations, and the author of this paper has also been able to confirm that cuttings are carriers of the disease. This was verified by taking 2 × 100 cuttings from a stock of carnations of the variety "Sidney Littlefield" where a *Phialophora* attack had been ascertained with certainty and caused one of the well-known "gaps" (Fig. 36). The cuttings were taken in a perfectly normal way, from apparently healthy plants near the "gap", and they were at once taken to the laboratory, where they were subjected to the ordinary sterilization process for "broth testing". Instead of being cut into the test tubes with nutrient broth, the small stem-pieces were put into test tubes with 2–3 ml PDA.

The test tubes were left at ordinary room temperature for 5 days, after which it was found that 2–5% of the tubes contained stem-pieces with a distinct growth of *Phialophora cinerescens*. The stem-pieces were left for observation for some 10 days more, but no more fungus-attacked stem-pieces could be demonstrated. From the 7 cuttings in question, cross sections of stem were made, and the presence of fungal hyphae in the xylem was demonstrated by means of microscopy. It is thus quite obvious that the disease is transferred from one stock of plants to another, inter alia, by cuttings, but it is not known for certain whether the disease is air-borne; there is, however, much to indicate that this is not the case. Here it may be mentioned that in England (WICKENS 1936) it is reckoned that conidia and conidiophores have never been observed on the wilted plants, and WICKENS furthermore reports that unwounded carnations are not attacked by the fungus after even artificial inoculation.

To elucidate the question of infection through the air, the author has tried if it would be possible to find wilting carnations with the conidial stage of *Phialophora cinerescens*, but, like WICKENS, he has arrived at the result that the fungus never sporulates on dying plants—in which the mycelium is probably not sufficiently ripe for it. In some dead carna-

Fig. 36. Culture of "Sidney Littlefield" attacked by *Phialophora cinerescens*. Note the typical "gap" in the stock of plants, which symptomatically might as well have been caused by a bacteriosis. Photographed 22.6.1956.

tions ("Canup's Pride") which had, in fact, been allowed to stand "too long" in the greenhouse, the author has, however, in two cases been able to demonstrate a cushion-like, heavily sporulating fungus-coating of *Phialophora cinerescens* on the stem (Fig. 37).

The question of the ability of the fungus to transfer infection by air-borne spores has not been further investigated, but such a way of infection is not likely. First of all, because the fungus seems to be unable to invade carnation stems above soil level; next, because so few conidia are produced on the plants; and, finally, because all investigated plants from the Danish nurseries have shown, without exception, that the infection

Fig. 37. Base of stem of the variety "Canup's Pride" attacked by *Phialophora cinerescens.* Note the white mould (hyphae and conodiophores) breaking out through the bark. ×1.3.

is introduced through the roots or the original cut-surface of the stem. On the other hand, the possibility of infection by conidia cannot be entirely excluded, unless the attacked plants—as generally done—are immediately removed from the healthy ones and destroyed.

In this connection it may further be pointed out that during the numerous isolations the present author has made, it was very often demonstrated that the basal part of the leaves on the infected stems of carnation also contained living hyphae of *Phialophora cinerescens.* These infected leaves may therefore be an important source of infection if torn off and left in moist soil.

C. Control

In accordance with the information stated above about finds of spore-bearing stems and infected leaves it is recommended—for safety's sake—that dying plants are removed from the crop immediately and burnt.

It is very important to burn the plants, as compost remains infectious for many years (see below).

The soil in which the diseased plants have grown is particularly infectious, as has been proved abundantly by investigations (Wickens 1935, Noordam 1948) and also in practice in Denmark and other countries. The soil therefore has either to be replaced completely with a fresh field soil or to be sterilized by means of chemicals or steam. Steam is most commonly used and has indeed proved effective, that is to say, when the "steaming" is done very carefully. In this connection it should be observed, however, that in practice it has been noticed that "fan-

mould" spreads faster in steamed soil than in fresh. Practical experience, furthermore, shows that renewal of soil and steam sterilization of beds laid out or in the ground in the greenhouse are of no avail, because neither can be carried out so thoroughly that infectious portions of soil (bits of roots) are totally removed or rendered completely harmless. English investigations (WICKENS 1935) as well as Dutch (NOORDAM 1948) bear out these observations, and NOORDAM has even ascertained that carnation roots attacked by *Phialophora* can, as a rule, be found in the soil at 25–50 cm depth, in some cases even at 75 cm; and neither steaming nor soil renewal is done to such a depth. NOORDAM also found that the fungus persisted at least for 4 years, but the present author has isolated *Phialophora cinerescens* from lots of tested carnations planted in "fresh" field soil upon which compost of dead carnations has been placed in one nursery 7 years, in another 16 years ago. At any rate the previous presence of these composts on the fields could be the only course of the broad attack of the fungus. If "fan-mould" occurs in beds laid out on the ground in a greenhouse, the only remedy seems to be to lay out the beds on so-called "raised beds"—of concrete, asbestos-cement, or some similar resistant and not very porous material. In practice it has been found that such raised beds and the soil thereon can be sterilized perfectly effectively with steam, if the steam pipes are submerged some way into the soil and the entire bed is covered with a "plastic sheet" sufficiently large to cover the surface and sides of the bed, and preferably its bottom as well; in this manner also the outer side of the bed is sterilized.

Since no exact investigations of the thermal death point of *Phialophora cinerescens* seem to have been carried out, and as this is a very important point in the planning of the control, the author has tried to determine the thermal death point of this fungus in naturally infected carnation stems.

The tests were arranged so that, after being sterilized superficially with "oxyspirit", 4–5 mm thick cross sections of naturally infected carnation stems ("Dark Red Sim") were placed on PDA in Petri dishes and incubated at 20–22°C.

After about 10 days, all the sections were completely grown over and through by *Phialophora*, which, incidentally, sporulated abundantly. The individual sections, with surrounding mycelium, were then cut out of the agar medium and transferred to other Petri dishes (one section of stem per dish) with fresh PDA (4–5 mm thick layer). These dishes were then incubated at 50, 60, 70, 80, 90, and 100°C, respectively, and

Table XVI. The thermal death point of *Phialophora cinerescens* in naturally infected pieces of carnation stems and its dependence on the time of treatment.

Time of treatment, minutes	Temperature in °C					
	50	60	70	80	90	100
5	+	+	+	+	+	−
10	+	+	+	+	−	−
15	+	+	+	−	−	−
20	+	+	−	−	−	−
25	+	−	−	−	−	−
30	+	−	−	−	−	−

+ growth within a week after treatment.
− no growth two months after treatment.

after 5, 10, 15, 20, 25, and 30 minutes' heating, respectively, three dishes from each "temperature" (incubator) were taken out. Thereafter each stem-piece was cut through with a sterile knife, and the agar and stem-pieces bearing conidia were then dragged over the fresh agar surface of other dishes (at 20°C), so that conidia, bits of mycelium and the stem-pieces were spread thoroughly all over the dish. After 2 months at 20–22°C the test was concluded and the fungal growth in the dishes investigated. The results are given in Table XVI, (above), from which it will be seen that *Phialophora* had survived in all the dishes at 50°C, even after 30 minutes' heating (steaming). In the dishes that had been heated to 60°C for 25 minutes, 70°C for 20 minutes, 80°C for 15 minutes, 90°C for 10 minutes, and 100°C for 5 minutes, no growth was found—even after 2 months—(the fungus had been killed). When the time of treatment was only 5 minutes shorter, the fungus, on the other hand, grew as well at the temperatures in question as it did in the check dishes, which had only been exposed to 20–22°C; in these dishes the fungal growth was distinct within a week.

The investigation thus seems to indicate that in steam sterilization of carnation beds the aim must be to take care to heat the "coldest" points of the bed to at least 70°C for not less than 20–30 minutes.

In practice, however, the treatment will probably not be as efficient as in the abovementioned laboratory test. Therefore, in order to ensure complete sterilization of the soil, the author is of the opinion that a somewhat higher minimum temperature of treatment—at least 80–90°C, and also a longer treatment, e. g. 30–45 minutes, will be necessary.

In connection with the problems relating to soil sterilization, also the question remains whether it will be possible to carry out a local sterilization of the soil after a single carnation or a few ones have been found to be attacked by "fan-mould"—or bacteriosis—and therefore removed. The problem arises especially for young or quite newly planted stocks where it is desired to plant new plants after the diseased ones, to preserve the stock complete in number. In these cases it will not be feasible to use steaming, and no satisfactory sterilization by formalin and mercury preparations has been obtained yet. The author has therefore tried whether it would be possible to use 8-hydroxy-quinoline sulphate also for this purpose, this substance in practice and in tests in the laboratory having proved to be particularly toxic to *Phialophora cinerescens* as well as other fungi and bacteria. Among other things the belowmentioned test showed clearly the toxic effect of the fungicide in question on *Phialophora*: Six Petri dishes with potato-dextrose-agar (PDA) (5 mm thick layer) were all thoroughly inoculated with conidia and bits of mycelium of *Phialophora cinerescens* by smearing the agar surface with pieces of infected carnation stems much overgrown by the fungus. Then a 9 mm wide hole in the middle of the plate was cut with a sterile cork-bore. As a check, sterile, distilled water was poured into the hole in one dish; into the other holes in the rest of the dishes were poured 0.25 ml of aqueous solutions of 0.13, 0.25, 0.5, 1, and $2^0/_{00}$ respectively, of 8-hydroxy-quinoline sulphate. After 15 days' incubation at 20–22°C the test was concluded and the dishes photographed (Fig. 38) from which is seen that at a concentration of $0.13^0/_{00}$ the fungicide has a marked effect; at 1 and $2^0/_{00}$ no growth of the fungus was observed at all.

The tests on the control of the fungus in practice by means of 8-hydroxy-quinoline sulphate aimed at killing the fungus in the soil by frequent watering of the localities in question with a $2^0/_{00}$ aqueous solution of the substance in question. It is not to be expected that these tests will be terminated these few years, but it can be said already that the carnations apparently stand watering with even $5^0/_{00}$ 8-hydroxy-quinoline sulphate, and they have suffered no harm from being planted in soil which had been mixed one week before with such a quantity of a $2^0/_{00}$ aqueous solution of 8-hydroxy-quinoline sulphate that the soil was fluid like oil. As already mentioned, it is yet too early to assess the tests, but there is a well-founded hope that fungi and bacteria will not survive such a powerful sterilization of the soil.

The question of excluding the attacked plants from the bed prepared for planting is of the utmost importance, as in the case of the bacterioses.

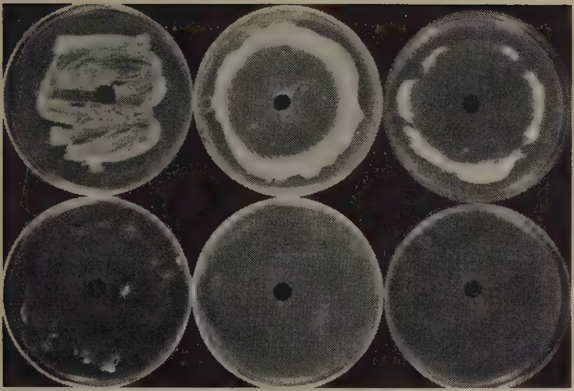

Fig. 38. The fungicidal effect of an aqueous solution of 8-hydroxy-quinoline sulphate on *Phialophora cinerescens*. Upper row from left: Check; next, treated with 0.13, 0.25 and, in the lower row from left, with 0.5, 1 and $2^0/_{00}$ 8-hydroxy-quinoline sulphate. Photographed 15 days after inoculation. ×0.3.

If this cannot be done, the costly soil sterilization or soil renewal will be of little avail. The author has therefore tried to find out whether, as in the case of the bacterioses, it would be possible to detect by some testing method the cuttings attacked by "fan-mould". The aforementioned test with the 200 cuttings was promising in this respect, and the method has now been introduced in two nurseries where testing for bacterial diseases is already in progress. The testing for "fan-mould" is quite simple: instead of only cutting stem-pieces down into "nutrient broth tubes", we also cut a few stem-pieces from each cutting into a test tube with 2–3 ml of PDA. The "agar tubes" are numbered like the "nutrient broth tubes" but are left in the laboratory at ordinary room temperature. Also in this instance it is too early to assess the results, but among the approximately 20,000 cuttings which have been tested so far the author has found a few sure cases of *Phialophora cinerescens*. It is thus a question whether the method indicates all attacked cuttings, but this low number of cuttings attacked by fungi is actually in good agreement with the number reckoned with in practice among newly planted carnations. Here it is not unusual that 2–3 plants out of 10–15,000 plants are infected.

In many other crops such a slight percentage of disease would appear to be highly satisfactory, but as far as carnations are concerned, it must be borne in mind that a single diseased plant in a newly planted bed—at any rate where the question is one of attack of "fan-mould"—or

bacterioses—may result in wilting of all the plants on the bed in the course of a year or two. It is therefore extremely important to find testing methods which are so sensitive that all the diseased cuttings can be separated off before insertion for rooting and planting, and it is the author's hope that the testing instituted will fulfil this very stringent requirement.

2. Collar-Fusariosis of Carnation

(*Fusarium dianthi* PRILLIEUX & DELACROIX,
Fusarium culmorum (W. G. SMITH) SACCARDO, and
Other Species of *Fusarium*)

A. Introduction

During the collecting of wilting carnations for use in the country-wide pathological analysis, isolated plants showing signs of incipient "wilt" were found here and there, especially in younger stocks of plants (3–18 months old). These plants were also included in the investigation, but the macroscopical examination of the cross section of the stems revealed plainly in some cases that the incipient wilting could hardly be due to bacteria or "fan-mould"; the stem rot observed was both too dry and too grey for that, and too limited longitudinally. In isolation tests from such plants originating from altogether 15 clones out of 147 (10.2$^0/_0$), two species of *Fusarium* appeared alternately, which in accordance with the descriptions of WOLLENWEBER & REINKING (1935) and WICKENS (1935) were determined to be *Fusarium dianthi* PRILLIEUX & DELACROIX and *Fusarium culmorum* (W. G. SMITH) SACCARDO.

These two species of *Fusarium* are described so fully by the aforesaid authors that a detailed description herein would appear to be superfluous, but a few characters should be mentioned. By cultivation on PDA the Danish isolates form an initially whitish, loose cottonwool-like mycelium (Fig. 31, p. 146), which soon—like the medium—assumes, a reddish and, later, a deep wine-red colour; thus the two cultures resemble each other to confusion. By microscopy they are somewhat more easily distinguishable. The conidia of *Fusarium culmorum* are generally broadly sickle-shaped and most often 5–6 septate, and as such they measure $36–65 \times 6–8 \mu$. Microconidia are produced sparingly or not at all. Chlamydospores are often formed intercalarily, both in mycelium and conidia. The conidia of *Fusarium dianthi* are as a rule somewhat slenderer than those of *Fusarium culmorum* and are most frequently 3–5 septate; they measure $30–70 \times 4–6 \mu$. Microconidia are produced in

large numbers and chlamydospores are often formed terminally, either singly or in short chains.

The species determination of the Danish isolates is not expatiated upon, since that would involve a very great investigation which would be entirely outside the scope of this paper. Moreover, the author is of the opinion that several different *Fusarium* species are able to produce "collar rot" in carnations and that the attacks depend in a lesser degree on the species than on the environment (wounding, humidity, etc.). This line of reasoning is in good agreement with indications from other countries, particularly England (WICKENS 1935, HOPKINS 1940), U.S.A. (GUBA 1945, BICKERTON 1942) and Holland (NOORDAM 1950), to the effect that other species of *Fusarium*, e. g. *F. avenaceum* (FR.) SACC., *F. oxysporum* SCHL., and others, may cause collar rot in carnation. On the other hand, according to the species concept of SNYDER & HANSEN (1941 & 1945) both *F. avenaceum* and *F. culmorum* are referred to one species only, viz. *Fusarium roseum* (LINK) SNYDER & HANSEN (1945). And according to the same authors, *F. dianthi* is a form of *Fusarium oxysporum*. This may be right from a pathological point of view, as so many species apparently are producing the same symptoms (no rule was determining when one species or the other were isolated). On the other hand, in a few cases both species were isolated from the same plant, and the cultural characters were definitely so distinctly different that the author does not like to accept the species concept of SNYDER & HANSEN until much more research on these species has been done.

Whether the carnation disease is produced by one species or the other, the symptoms seem in all cases to be those of a general wilt disease the actual starting point of which is a more or less deep-seated rot in the collar of the plant, and the author would therefore propose that the disease is called "collar-fusariosis of carnation" (in Danish: "Nellikens Rodhals-Fusariose").

B. Description of the Disease

History and distribution. *Fusarium dianthi* has previously been observed in Denmark causing carnation wilt (LIND 1913, The Danish Plant Pathological Research Station 1922, 1931, 1932), whereas it has not been possible to find any Danish indications of attacks of *Fusarium culmorum* in connection with wilted carnations; the fungus is, however, by no means unknown in Denmark, where it has been observed, inter

alia, on oats, rye and wheat[1]). Collar-fusariosis of carnation is, otherwise, spread the world over and has now, besides from Denmark, been reported from no less than 19 countries, viz.:

Argentine (Carera 1940), Austria (Schmidt 1952), Belgium (Wollenweber & Reinking 1935), Bermuda (Waterston 1939), Czechoslovakia (Zimmermann 1924), England (Dowson 1929, Wickens 1935, White 1935, 1936, 1938), Finland (Linnasalmi 1952), France (Fron 1936, 1937, Moreau 1953, 1957), Germany (Wollenweber & Reinking 1935), Greece (Aganostopoulus 1933), Hawaii (Hendrix & Murakishi 1950), Holland (Noordam 1950), Italy (Montemartini 1924, Bongini 1930, Calvino 1937, Camici 1948), New Zealand (Cunningham 1931), Norway (Roll-Hansen 1950), South Africa (Wijers 1937), South Rhodesia (Hopkins 1940), Uganda (Bickerton 1942), U.S.A. (Bickerton 1942, Guba 1945, Thomas 1948).

Symptoms and etiology. The disease attacks carnations of all ages. In cuttings or quite young, rooted plants a brownish, wet and soft rot arises in the lowermost portions of the stems; the roots—if formed at all—decay completely. The rot is as a rule confined to the lowermost 5–10 cm of the plants but may extend right up into the top of young plants. The attacks involve more or less sudden wilting of the plants, which at first "grow poorly" and later assumes a non-characteristic greenish-grey colour.

When young diseased plants are cut through, a distinct, brown discoloration of the pith is seen, particularly in cases of comparatively light attacks; but if the attack is more advanced the whole cross section of stem may be brown-rotted; sometimes the stems may even be hollow.

Attacks on older plants also involve a non-characteristic wilting of the whole plant, but by a transverse cut of the collar (5–10 cm above soil level) the attacked portion of the stem is found not to be wet-rotten as in the cuttings; on the contrary, it is dry and fibrous and has a characteristic brownish-grey colour. In older plants the rot of the stem tissue may—as in the case of quite young plants—spread over smaller or larger parts across the stem right from the outer bark to the middle of the pith. The extent of the diseased tissue lengthwise in the plant is remarkably confined and extends but rarely beyond about 5–20 (30) cm up the plant. The roots of attacked plants are often found to have decayed completely; but they may also be fresh, especially if attacked tissues do not reach down into the soil!

[1]) Herbarium material. Dept. Plant Pathology; Royal Vetr. & Agric. College, Copenhagen.

Altogether, the Fusaria do not seem to play any important part in the stocks of Danish carnations. This is also apparent from the low number of cases that appeared during the isolation tests as well as from the fact that *Fusarium* was isolated only from stems that had been wounded somehow at the base. Abnormally low pinching of the young plants, wounds produced mechanically, burning by too fresh and clammy manure, etc., in conjunction with too high a relative humidity of the air ambient were frequently found to be the primary causes of the *Fusarium* attack. This is in fair agreement with the fact that the Fusaria are generally considered wound parasites and are frequently isolated from the outer (bruised) stem parts, more rarely from pith or vascular tissues.

As Danish Fusaria will not extend very high up in the plants normally used as mother plants, there is hardly any risk of the disease being transferred from one stock of plants to another by cuttings containing mycelium in their stems. On the other hand, air and soil are the most important ways of infection of the fungus, and therefore cuttings may very well carry conidia on leaves and stems, and in this way the cuttings may, after all, act as carriers of the disease.

Wickens (1935) has carried out some infection tests with infected sand and wounded and unwounded cuttings and plants, and he arrived at the result that sand (soil) containing infectious matter (conidia, chlamydospores, hyphae, etc.) from *Fusarium* is extremely infectious for cuttings and other wounded carnation plants, but that unwounded plants are difficult to attack, even by *F. dianthi*. This is in excellent agreement with the author's observations, according to which *Fusarium* will attack carnations through wounds, and then only if the surroundings are very humid. The infectious matter (conidia) is found in any stock of plants because the fungus lives as a saprophyte on wilted leaves or stems of carnations. The author has often placed fresh carnation stems in a moist chamber and, so far, has always found that the stem-pieces were overgrown by *Fusarium* mycelium, even when the stem-pieces were taken from healthy plants.

C. Control

From the above it will be realised that active control measures against *Fusarium* attacks in older stocks of plants are of no avail; nor are they practicable in the often very dense stocks. For such plants the control consists in attempting to avoid direct wounding of the stems and in keeping the air and the surface of the soil as dry as possible.

As far as the cuttings are concerned, it is a prerequisite that they are propagated in separated propagation houses and that these houses as well as the benches and the sand are sterilized completely before each stock of plants. Should *Fusarium* attack nevertheless occur on the propagation bench, active control in the form of frequent watering with fungicides may come into consideration. From France (FRON 1937) watering with 0.1$^0/_{00}$ 8-hydroxy-quinoline sulphate is recommended, and the new "Ferbam", "Ziram", and "Zineb" preparations have proved effective in Danish nurseries. From U.S.A. GUBA (1949) states many commonly used fungicides being toxic to *Fusarium*, but SVENDSEN (1957) recommends especially a new methyl mercury compound "Panogen".

Chapter V

Review of Differentiation of the Four Carnation Wilt Diseases Described

Since it has been demonstrated by the investigations recorded in the foregoing that "wilt disease" in Danish perpetual-flowering carnations may be due to at least four different causes, namely two bacterioses and two mycoses, all of them, largely, resembling each other to cause confusion, it may be appropriate to describe, in conclusion, the symptomatic differences which, after all, exist between the diseases, to make comparatively easy differentiation of them possible in practice.

On the basis of the experience gained in the present investigations, the author has tried to list the symptoms characteristic of the four diseases in a key affording a ready review of the differences. It should be noted, however, that this key is applicable only to wilting (partially wilted) plants and only to plants that are at least six months old. Wilt diseases in cuttings and carnations which have only recently been planted out are so much alike that isolation tests on PDA provide the only means of making a sure diagnosis.

To find the cause of a wilt disease in carnations, it is necessary first to pull the wilting plant with its root out of the soil and examine some transverse sections of the stems, 20–40 cm above the root. The cut-surfaces produced should then be examined macroscopically or through a hand lens, and if the vascular tissues in some of the stems appear as a sharply defined, brown—often dark brown—continuous ring within the bark (Fig. 39), and the root system and collar are found to be well preserved without easily visible discoloration, one may be fairly sure of being faced with an attack of "fan-mould of carnation". Moreover, this disease makes many of the top leaves of six months old and older plants irregularly red-mottled, a symptom which has not been observed in connection with any of the other wilt diseases. When discs of stem are cut off under aseptical conditions and placed on PDA, white hyphae

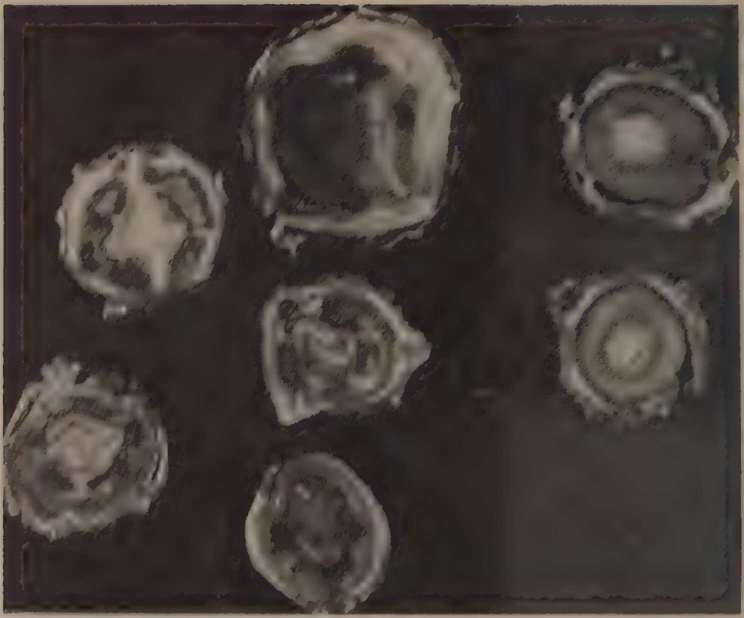

Fig. 39. Cross section of basal portion of stems of the variety "White Sim", photographed 26.6.1956. The 3 discs of stem to the left display irregular brownish and water-soaked "blotches" in the vascular tissue, caused by attack of Pectobacterium parthenii var. dianthicola (the symptoms may well be confused with those of bacterial stem-crack of carnation). The two discs of stem to the right show a uniform, sharply defined, brown discoloration of the vascular tissue due to attack of Phialophora cinerescens. ×2.5.

from the xylem will appear in 2–4 days if this disease is present (Figs. 32 & 33, p. 148) but the growth of the fungus on the medium is so slow that even after one week (at a temperature of 18–22° C) the culture will measure no more than 1–1.5 cm in diameter. In a couple of weeks the culture will turn grey or blackish-grey and the diameter of the culture will not exceed 2–3 cm.

If the cross sections of stem at the indicated distance from the root display nothing abnormal, or if more or less irregular, brown spots are seen in the xylem or in the pith, the lowermost 20 cm of the stem ought to be examined by slitting and, further, transverse cutting, as the question may then be one of attack by bacterioses or by fusarioses.

On cross sections of the lower portions of stem the bacterioses appear as one or more irregular blotches which, plainly, have arisen from the

xylem but gradually have spread outwards to the bark and inwards into the pith and have a water-soaked appearance in the margin (Fig. 39). On cross sections there is but little difference between the pathological pictures of the two bacterioses, but it should be observed that bacterial stem-crack of carnation often destroys the vascular tissue to such an extent that holes arise in the stems, which is rarely seen in connection with bacterial stunt of carnation. Furthermore, the lower parts of the stems attacked by "stem-crack" are often beset with deep and long cracks, and the xylem is very sticky. The characteristic stunted shoots produced by bacterial stunt of carnation, in conjunction with the non-crack-beset stems, no doubt constitute the safest symptom by which this disease can be distinguished from bacterial stem-crack of carnation, which generally leaves the top shoots perfectly normal.

In cultures on PDA—and when cross sections of stem are aseptically cut off and placed on PDA—the two bacterioses are very easy to distinguish from each other. In about 6 days the pathogen of bacterial stem-crack forms entire-margined, at first cream-coloured, later brown—dark-chocolate-brown colonies, whereas bacterial stunt in the same period on the same medium (PDA) forms greyish colonies with very distinct lobate margins. (Figs. 24 & 25, pp. 112–113).

When the basal portion of the stem (the lowermost about 20 cm) is transversely cut through, the collar-fusariosis is identifiable as a very irregular, brownish-grey dry fibrous rot, which is not particularly bound up with the vascular tissue but more often it attacks the pith or the bark. Further, it is a characteristic feature that the discoloured portion in the stem extends only 5–20 (30) cm up the stem, that the transition to healthy tissue is sharply defined, and that the rot is fibrous and as a rule springs from wounds on the base of the stem.

In cultures on PDA, *Fusarium dianthi* and *F. culmorum* mycelia, which are at first white, loose and cotton-wool-like, reach a diameter of about 3–4 cm in 3–4 days (Fig. 31, p. 146) and turn dark wine-red in a week's time.

Key

to differentiation of four wilt diseases of carnation.
(Determination to be made on wilting plants).

I. Vascular tissue in cross section of stem (20–40 cm above the root) always uniformly discoloured, brown stained and sharply defined from the pith (Fig. 39). The discoloured tissue is seen as a brown "ring" (or parts thereof) inside the bark, which is not soft and rotten. The top leaves, particularly those of older plants, irregularly red-mottled. Root system generally well

preserved. When discs of stem are aseptically cut off and placed on PDA, white hyphae appear from the discoloured vascular tissue in 3–4 days (Figs. 32 & 33); during this time the fungus extends, at most, 1–2 mm out on the medium.

.... Fan-mould of carnation (*Phialophora cinerescens*)

II. Vascular tissue in cross section of stem (20–40 cm above the root) not uniformly brown-stained (no brown "ring" inside the bark). In vascular tissue as well as in pith and bark one or more irregular, brownish spots or blotches are visible (Fig. 39). Top leaves never red-mottled. Root system decayed more or less. When discs of stem are aseptically cut off and placed on PDA, either slimy bacterial colonies (A) or rapidly growing fungal mycelium (B) appear.

A. Cross section of stem (20–40 cm above the root) beset with few or several brownish, irregular blotches having a water-soaked margin and plainly starting from the vascular tissue (Fig. 39). When discs of stem are aseptically cut off and placed on PDA, slimy bacterial colonies appear.

a. Top shoots normal. Lower portion of stem often beset with deep longitudinal cracks (Figs. 3 & 21). Vascular tissue very sticky when the bark is peeled off. Bacterial colonies on PDA dark brown after 6 days.

.... Bacterial stem-crack of carnation (*Pseudomonas caryophylli*)

b. Top shoots often found to have abnormally short internodes (Figs. 20 & 21). Cracks in stems rare, and never deep; stem base often somewhat thickened. Bacterial colonies on PDA after 6 days greyish with coralloid margins (Figs. 24, 25, & 30).

Bacterial stunt of carnation. (*Pectobacterium parthenii* var. *dianthicola*)

B. Cross section of stem (5–20 cm above the root) show a more or less extensive brownish-grey, dry and soft, fibrous rot, which may have its starting point in the bark as well as in the pith, rarely in the vascular tissue. The discoloured portion is largely confined to the collar, at most 15–20 cm up the stem. When discs of stem are aseptically cut off and placed on PDA, white cotton-wool-like fungal mycelia appear, which measure about 3–4 cm in diameter after 3–4 days (Fig. 31). The mycelium and the medium gradually assumes a wine-red colour.

.... Collar-fusariosis of carnation (*Fusarium dianthi* and others)

Summary

Chapter I. Bacterial Stem-Crack of Carnation.
(*Pseudomonas caryophylli* (BURKH.) STARR & BURKH.).

In October 1953 the Department of Plant Pathology of the Royal Veterinary & Agricultural College in Copenhagen received from a Copenhagen nursery a couple of hundreds of carnation cuttings of the variety "Harvest Moon". Although the cuttings had a healthy appearance, most of them died, assuming a greenish-grey discoloration when planted out. A closer examination revealed that the vascular tissue had brownish-yellow discolorations in the form of stripes, originating from bacteria-filled and partially decomposed vessels. From these *Pseudomonas caryophylli* (BURKHOLDER) STARR & BURKHOLDER was isolated, which in 1942 was described as a new species of bacterium and as the cause of the carnation disease called "bacterial wilt of carnation", which had recently arisen in the U.S.A. This common name, however, is not characteristic of this one carnation disease, and the author, therefore, has proposed the more descriptive name: "bacterial stem-crack of carnation" (in Danish: "Nellikens Sprækkebakteriose").

Bacterial stem-crack of carnation was first observed in perpetual-flowering carnations—inter alia, the variety "King Cardinal"—in the state of Washington, U.S.A. (L. K. JONES) in 1941, and the following year the bacterium was described by W. H. BURKHOLDER under the name of *Phytomonas caryophylli*, which STARR & BURKHOLDER the same year (1942) changed into *Pseudomonas caryophylli*. Bacterial stem-crack of carnation has since been found widespread in the U.S.A., and in the years 1953–55 the author has found it in Denmark, England, Holland, Germany and Sweden, into which countries it has probably been imported directly or indirectly by cuttings from America.

Bacterial stem-crack of carnation is a pronounced vascular bacteriosis destroying the vessels (Figs. 1 & 2) and causing the plants to wilt suddenly, assuming a general greenish-grey discoloration. On cross sections of the diseased stems the infected tissues appear as more or less uneven,

brownish spots which are only seen in the vascular tissues (Fig. 39). If the bark is peeled off the diseased tissues appear as shorter or longer brownish streaks which sometimes may be seen right up in the top of the plants. It is characteristic that the infected vascular tissue is very sticky. The disease is transmitted by cuttings, and if the infection is comprehensive, root formation in the propagation bed is prevented and the cuttings will die. The bacteriosis may also be less comprehensive—i. e. few vessels are infected—and even develop so slowly that the cuttings may form roots; in such slightly infected plants the bacteriosis will produce deep cracks in the carnation stems, particularly in younger plants (Figs. 3 & 21). Slightly infected plants may grow normally for a long time without showing any sign of disease, but since, after all, they harbour disease-producing bacteria in some of their vessels, there is a great risk of taking infected cuttings from these "carriers" in the cultivation period. When older carnation plants are killed by bacterial stem-crack, this happens—as in the case of the younger plants—with a not particularly typical wilting while a greenish-grey discoloration is becoming evident, but it is characteristic that the root systems of the plants have decayed more or less and that the brownishly discoloured vascular tissue is very sticky when the bark is peeled off. Under Danish climatic conditions the disease seems to spread little among planted-out carnations, due to the low soil temperature in this country.

Isolation of *Pseudomonas caryophylli* is effected easily by cutting out from carnation stems small pieces of brownishly discoloured vascular tissues and putting them into sterile water, after which the suspensions are streaked on to potato dextrose agar (PDA). After 2 days, barely visible, cream-coloured colonies appear, which become chocolate-coloured and tough after 6 days at 27°C. A random isolate (No. 83) was chosen for infection tests. Inoculation of bacterial slime in stabs produced artificially in the stems of young, firmly rooted carnation plants resulted in deep longitudinal cracks in the stems after one month and many vessels were destroyed. From these vessels *Ps. caryophylli* was re-isolated. Infection tests, performed by inoculating the plants in different ways through wounded roots, showed that the bacteria damaged the root system of the plant to such an extent that the carnations wilted in 4–6 weeks while turning greenish-grey, but without developing cracks in the stems. Simultaneously, it was ascertained that carnations may adopt the bacteriosis by being planted in soil which contains the bacteria.

In the first 10 days after being taken off, an unrooted cutting will absorb about 0.6–1.8 ml water a day, and thereby it is able, after 2 days,

to take up bacteria about 3–9 cm up the vessels of the stems. The vessels proved to consist of long, straight and nearly continuous tubes, 10–18 μ broad (Fig. 7). Thus, the possibilities of establishing infections are particularly favourable when cuttings are placed in liquids or aqueous media to which *Ps. caryophylli* has been added in one way or another.

Pseudomonas caryophylli is a slightly curved, rod-shaped, Gram-negative bacterium measuring about $0.5–0.7 \times 1.4–3.0\ \mu$ (Fig. 8). Further, the bacterium has 1–5 polar or bipolar flagella which it is very difficult to stain with aniline dyes according to ordinary staining methods; but electron micrography has proved excellent for demonstration of the position and number of the flagella (Figs. 8 & 9).

The bacterium will form small yellow colonies on nutrient agar, but grows poorly on this medium, where it is only able to subsist for about 6 days. In nutrient broth it will grow abundantly, however, PDA is an excellent agar medium, on which colonies are formed which are at first cream-coloured, pulvinate and shiny, but after 5–6 days at about 27°C they become dark brown with a narrow, cream-coloured margin. Gelatin is not liquefied.

By cultivation on PDA the optimum temperature for the bacterium has been determined to be 30–37°C, the minimum temperature about 10–15°C, the maximum temperature 45–46°C, and the thermal death point 53°C. In sterile soil at 10°C the bacterium keeps well for one year or more. In liquid, inorganic nutrient media (indicator: bromthymol blue) at 28–30°C the following carbohydrates and alcohols are fermented without formation of gas: glucose, fructose, xylose, galactose, mannose, saccharose, sorbitol, arabinose, raffinose, mannitol, and glycerol. Lactose and maltose are fermented slightly in 3–4 weeks. Salicin, dextrin and inulin are not fermented. Starch is not hydrolized, and production of indole and hydrogen sulphide could not be demonstrated, but a slight formation of ammonia and nitrite was found. Both the methyl-red test and the Voges-Proskauer test were negative. Neutral litmus milk slowly becomes slightly alkaline and thereby assumes a dirty bluish-green colour, and after 2 months a violet-coloured clearing of the uppermost layers of the medium develops.

As dissemination of bacterial stem-crack of carnation takes place mainly during the propagation of cuttings, the control should primarily aim at preventing the transfer of the bacteria from one cutting to another. The cuttings therefore must not be placed in water before insertion in the sand bed; and if liquid rooting stimulants are used, these should be kept sterile by the addition of 0.3–0.4$^0/_{00}$ 8-hydroxy-quinoline sulphate;

treatment with rooting stimulants in the form of powder is recommended.

During the stay of the cuttings in the propagation bed a direct control of the bacteriosis is hardly practicable, but the sand and the bench materials are infectious following an infected stock of plants; the sand and the bench materials should therefore always be renewed entirely or be thoroughly sterilized. Washing of boards with "Aretan" (an organic mercury preparation) or similar disinfectants are insufficient; wood should therefore be avoided in connection with the propagation of cuttings.

Tests with the use of aqueous solutions of "Aretan" and 8-hydroxy-quinoline sulphate in different concentrations as systemic-acting bactericides had the result that "Aretan", even in an 0.35$^0/_{00}$ concentration proved to be lethally toxic to carnation cuttings; 0.5$^0/_{00}$ 8-hydroxy-quinoline sulphate was not toxic to the cuttings and could be absorbed about 5–6 cm up the stems in 2 days but was unable to kill all the bacteria in the vessels.

Control of the bacteriosis by antibiotics has been attempted. Streptomycin (Fig. 11), terramycin (Fig. 10), and Agri-mycin 100 (Fig. 12) had good antibiotic effects on *Ps. caryophylli*, in vitro as well as in vivo, but as Agri-mycin 100 had the best effect and is also specially designed for the control of plant diseases, particularly the effect of this substance on carnation was made the subject of further investigation. In this it was found that Agri-mycin 100 could be absorbed in carnation cuttings and that the strongest concentration used, 5.6$^0/_{00}$, was taken right up into the top in the course of 1 day (Fig. 13); after an absorption time of 2 days, also Agri-mycin solutions of 2.8 and 1.4$^0/_{00}$ were found to be taken right to the top of the stems. The effect of 1.4 and 0.7$^0/_{00}$ solutions was considerably less than that of the stronger concentrations and not certain in the topmost portion of the stems. An Agri-mycin solution (5.6$^0/_{00}$) absorbed by carnation cuttings caused all the tissue-portions of the stems—with the exception of the epidermis—to be "impregnated" with the substance, and the antibiotic effect lasted for about 4 weeks. Used in 5.6 or 2.8$^0/_{00}$ strength, the solution prevented root-development in carnation cuttings, and only a concentration of 0.007–0.07$^0/_{00}$ allowed approximately normal root development. Since such low concentrations had practically no bactericidal effect on *Ps. caryophylli* in the stems, and since Agri-mycin 100 could not be absorbed through the roots, on which it furthermore had a very toxic effect, control by the antibiotics tested had to be considered impossible for the present.

As direct control measures against bacterial stem-crack of carnation seemed to be useless, it was tried, by microscopic examination of cross sections of stem of cuttings (the "microscopy method") to sort out the

diseased individuals, so that they might be discarded. This method of investigation proved unreliable because quite young infections cannot be seen in the vascular tissue of the stems, and the "microscopy method" should not be used.

For demonstration of the disease in cuttings, biological examination of the sterility of the inner tissue of the stems of the cuttings is more reliable than the "microscopy method", but the direct placing of discs of stem on PDA in Petri dishes (the "agar method") is not infallible; bacterial growth from discs with but few infected vessels could not be demonstrated in a few cases.

Very certain results were obtained by putting small pieces of stem in water (the "suspension method") or in nutrient broth (the "broth method"), with subsequent streaking of these suspensions on to PDA; and for mass examinations of the state of health of carnation cuttings a relatively quick and inexpensive check method has been elaborated.

This method, called in practice the "broth method", is carried out as follows: the lowermost internode or the lowermost 3–4 cm of the stem of the cutting, which has not at all been in touch with water, is washed very carefully with $70^0/_0$ alcohol + $5^0/_{00}$ 8-hydroxy-quinoline sulphate ("oxyspirit"), after which the cutting is placed in a $5^0/_{00}$ aqueous solution of mercuric chloride for 20–30 seconds. Thereafter the mercuric chloride is washed off in "oxyspirit" and the base of the stem is wiped once more with "oxyspirit", which eventually is burnt off. From the superficially sterilized internodes the lowermost about 1 cm piece of stem is cut off with a pair of flamed scissors and discarded. Next, three pieces of stem, 5–10 mm long, are cut off directly into test tubes with about 5 ml nutrient broth + $1^0/_0$ glucose (Fig. 14), whereupon the cutting is cut clean (made ready for insertion in the sand bed) with a sterile razor blade and placed in a numbered test tube with boiled tap water (Fig. 15). The test tube with the matching pieces of stem is marked with the same number and incubated at 26–27°C (Fig. 16). After 4–6 days, the tubes in which the bacterial growth has been ascertained are taken out, and cuttings with the same numbers are discarded. The rest of the cuttings should be healthy, but a few infected cuttings may now and then pass the inspection. Therefore it is necessary to propagate the tested cuttings separately in small pots (one cutting in each pot) placed in chicken wire netting, or on such netting on pebbles on the propagation bench (Fig. 28), so that the bacteria cannot be spread during the rooting period. If this way of propagation is repeated with two or three consecutive generations of carnations, it should be possible to eradicate the disease completely within 6–18 months.

Chapter II. Bacterial Stunt of Carnation.

(*Pectobacterium parthenii* var. *dianthicola* var. n.).

In Denmark, bacterial stunt of carnation was first observed in cuttings of the variety "Salmon Sim" in the autumn of 1955 and in mother plants of the variety "Lucky Strike" in the spring of 1956, but on both occasions it was supposed to be attack of *Pseudomonas caryophylli* (BURKH.) STARR & BURKH., partly because the attack was so similar to that of this latter bacterium and partly because at that time no other vascular bacteriosis of carnation had yet been described. Isolation and infection tests have revealed, however, that the recently observed "wilt disease" of carnation is due to attack by a new species of bacterium.

In Holland, this new disease of carnation was first observed in 1951 (BAKKER & SCHOLTEN 1955), but also there it was confused with attack of *Pseudomonas caryophylli*. The disease was observed almost simultaneously in England (LELLIOTT) and Danmark (the author) in the autum of 1955, and in April 1956 it was described by LELLIOTT under the name of "slow wilt of carnations". LELLIOTT determined the new carnation pathogen as a strain of *Erwinia chrysanthemi* BURKHOLDER et al. but did not give this strain any name.

On the basis of cultivation and infection tests with isolates of *Erwinia carotovora* (JONES) HOLLAND, *E. atroseptica* (VAN HALL) JENNISON, *E. chrysanthemi* BURKHOLDER et al., *E. carotovora* f. sp. *parthenii* STARR, and one Dutch, one English and two Danish isolates of the new carnation pathogen, the author of this paper has come to the conclusion that it is not identical with any of the aforesaid bacteria and that *E. carotovora* and *E. atroseptica* can be distinguished clearly from each other and from other Erwinias. The author refers the said bacteria to the genus *Pectobacterium* WALDEE and raises *Erwinia carotovora* f. sp. *parthenii* STARR to a species of this genus. *E. chrysanthemi* BURKHOLDER et al. and the carnation pathogen are set up each as a variety of this species. Consequently, the following names are proposed for the respective bacteria: *Pectobacterium carotovorum* (JONES) WALDEE, *P. carotovorum* var. *atrosepticum* (VAN HALL) DOWSON, *P. parthenii* (STARR) comb. n., *P. parthenii* var. *chrysanthemi* (BURKHOLDER et al.) comb. n., and for the new carnation pathogen: *Pectobacterium parthenii* var. *dianthicola* var. n., simultaneously, it is proposed that the disease should be named "bacterial stunt of carnation" (in Danish: "Nellikens Kortskudbakteriose").

A review of the differentiating characters of the isolate types and a key to determination of the five bacteria are given pp. 124 & 130.

Like bacterial stem-crack, bacterial stunt of carnation causes the attacked plants to wilt. However, if the infection (the destruction of

the vessels) is of a comparatively small extent, the diseased plants may live for a long time, but they will often produce stunted shoots (Figs. 20 & 21) with leaves that are a little narrower than normal. By production of meristematic cells (callus) around the infected vessels (Fig. 19), the plant will try to replace the diseased tissues, and this growth causes a thickening of the base of the stem, but deep cracks—as produced by bacterial stem-crack—have not been observed.

When cross sections of the diseased stems are made, the infected tissues appear as smaller or larger, uneven, light brown and towards the edges somewhat water-soaked patches or spots, which are limited to the vascular tissues (Fig. 39); if these are exposed by peeling off of the cortex of the stem the diseased parts will be seen as streaks along the stem; some streaks are often found right up in the upper parts of the plant.

The disease is, in general, transmitted from one crop to another by cuttings taken from infected mother plants. From the infected cuttings the bacteria are spread to the others while they are placed in water, solutions of rooting stimulants, etc. and in the propagation bed during the rooting period ("primary infection").

If the cuttings take up many bacteria, the cuttings are apt to wilt within a short time without forming roots, and the base of the stem may be soft-rotted. On the other hand, if only a few vessels are invaded the cuttings form many roots, and so they are mixed with the healthy ones and planted out together with these. After this, the diseased plants usually develop characteristical, stunted shoots and a somewhat thickened base of the stem, but some slightly infected plants may also grow for a long time showing no symptoms of disease. In such plants the bacteria may reach the very top of the plant and so there is a great risk of taking infected cuttings from these healthy-looking plants.

In seriously infected plants, especially the elderly and "secondarily" infected plants, the root system is often so much disintegrated that the bacteria but rarely penetrate more than 20–40 cm up the stems before the plants die.

When the vascular tissues in the roots are attacked, the roots usually disintegrate, and so the bacteria are mixed with the water in the soil. The possibility of root infection is thereby brought about and this way of infection ("secondary infection") actually seems to be the most important one among plants planted out. When the soil is contaminated after an infected crop, the disease may be transmitted from one crop to another in this way as well.

Bacterial stunt of carnation seems to be highly liable to spread in older (1 to 3-year-old) stocks of plants, which may presumably be

accounted for by the comparatively low optimum temperature of the bacteria.

Isolation of the bacterium is easily effected by aseptically cutting out small pieces of brownishly-discoloured vascular tissue, which are put into a few millilitres of sterile water, from which streaking on to PDA in Petri dishes is then effected after 4–6 hours. On this medium the bacterium forms greyish, roundish colonies, which, after 2–3 days, appear with very distinctly lobate margin and an umbonate centre, which is light brownish-yellow in transmitted light. A single isolate (No. 98) was chosen for further investigation.

Young carnation plants of the varieties "Lucky Strike" and "William Sim" which were inoculated with isolate No. 98 (the original isolate) in stabs in the stems produced stunted shoots in 2–6 months and wilted eventually while assuming a greenish-grey discoloration. Re-isolation from these plants (isolate No. 99) and re-inoculation confirmed that the carnation disease was caused by the newly discovered "carnation-bacterium".

Infection tests carried out by putting diseased cuttings as well as healthy ones in the same medium and box proved that the bacteria during the rooting period are able to pass from diseased cuttings to healthy ones, thus spreading the disease.

The isolated bacterium is Gram-negative, rod-shaped with rounded ends, and has 1–14 flagella arranged peritrichously; it produces neither capsules nor spores. The bacterium measures $0.7\text{–}3.5 \times 0.5\text{–}0.9\,\mu$ (Figs. 22 & 23).

Gelatin is liquefied in a saccate manner. On nutrient agar, dirty-white, slightly convex colonies with somewhat lobate margins are formed. On PDA it has a very characteristic smell and larger colonies are formed than on nutrient agar; on PDA the colonies become distinctly lobate (Fig. 24 & 25). Sterile potato plugs are completely decomposed ("soft rot") in a couple of days. On Endo agar, pink colonies appear; on eosin-methylene-blue agar, the colonies are likewise reddish but mucoid, and many "violet spots" (eosin-methylene-blue compound) are visible in them. On desoxycholate agar, colonies are slightly pink or yellowish. The optimum temperature for the bacterium was determined to be 25–27°C, the minimum temperature about 5–10°C, the maximum temperature was about 35–36°C, and the thermal death point 49–50°C. The bacterium will remain viable on nutrient agar for 4 months at minus 6°C, and in sterile soil at 10°C for two months.

In inorganic liquid medium with bromthymol blue as indicator the following carbon compounds were fermented at 26–27°C with formation

of acid and gas: arabinose, xylose, glucose, galactose, mannose, fructose, saccharose, raffinose, inulin, mannitol, and salicin. Glycerol, rhamnose, aesculin, and ethanol were fermented slowly without formation of gas. Lactose and, especially, maltose were fermented very slowly. It seemed as if dextrin, erythritol, sorbitol, dulcitol, Na-hippurate, and NH_4-lactate could be utilized by the bacterium to some extent, but the colour of the media was unchanged after 3 weeks. In Na-acetate, Na-citrate, Na-succinate, Na-tartrate, and Na-malonate, alkali was produced. $1^0/_{00}$ aesculin in nutrient broth was stained jetblack. In nutrient broth + $5^0/_0$ NaCl no growth.

Starch was not hydrolized. Ammonia and H_2S were not formed, but indole was produced and nitrate was reduced to nitrite. The methyl-red test was positive after 2 days, negative after 5–6 days. The Voges-Proskauer test was constantly positive. After 3–4 days, neutral litmus milk became bluish-grey, and was turned into soft curd in 6–8 days; litmus was reduced, and a top layer of about 0.5 cm depth of the medium was peptonized.

As bacterial stunt of carnation is very often transmitted by the contaminated soil and bench materials, the control of the disease must be directed so that soil sand etc., and also wooden boards and the like, are thoroughly steamed or exchanged with quite new materials.

When new crops are commenced it is necessary to plant entirely healthy cuttings.

Until now cuttings originating from mother plants tested according to the "broth method" (see p. 175) have been taken for healthy, but in May 1957 it was shown that a few infected cuttings now and then could pass the examination. To prevent the spread of the disease in the propagation bench it is necessary to root the tested cuttings separately in small pots (one cutting in each pot) placed in chicken wire netting, or on such netting on pebbles (shingle) on the propagation bench (Fig. 28) and keep the young plants in pots on shingle. If this way of propagation is repeated with two or three consecutive generations of carnations, it should be possible to eradicate the disease completely within 6–18 months.

Chapter III. The Incidence of the Carnation Bacterioses—as well as of *Phialophora cinerescens*, *Fusarium dianthi*, and *Fusarium culmorum*—in Denmark.

As a conclusion of the work with bacterial stem-crack of carnation and bacterial stunt of carnation, an investigation of the incidence of

the two bacterioses in 53 Danish carnation nurseries was made. For this purpose wilting carnations were selected from altogether 147 clones (stocks of plants) distributed in 31 varieties (Table XV). Each individual plant was examined macroscopically and microscopically, and it was tried to isolate both fungi and bacteria from all the plants. Discs of stem were aseptically cut off from diseased plants and placed on PDA in Petri dishes (Figs. 30, 31, 32, & 33) or put into sterile, distilled water, from which streaking on to PDA was done.

The pathological analysis of the stocks of Danish carnations revealed that bacterial stem-crack occurred only in a single clone, and the almost complete eradication is believed to be due to the very effective control measures—including "nutrient broth testing" of cuttings—which were instituted immediately as well as to the poor ability of the bacterium to stand soil temperatures below 10–15°C.

Bacterial stunt of carnation was found in 26.5% of the clones examined, and it is presumed that the disease has become so widespread partly because this bacteriosis has remained unknown for a relatively long period and partly because the conditions for the growth of the bacterium are well accommodated by the climate prevailing in the stocks of Danish carnations.

In the pathological analysis of the stocks of Danish carnations it was, furthermore, ascertained that 54.4% of the diseased clones were attacked by *Phialophora cinerescens* (Wollenweber) van Beyma, a fungus which had not previously been observed in Denmark but is known in England, Germany and Holland. This fungus seems to be the main cause of the "wilt disease of carnation" in perpetual-flowering carnations in Denmark. The disease is further mentioned in Chapter IV.

From 10.2% of the clones, two *Fusarium* species were isolated: *Fusarium dianthi* Prill. & Del. and *Fusarium culmorum* (W. G. Sm.) Sacc., but these fungi seem to be of little importance in the stocks of Danish carnations. See also Chapter IV.

From 8.2% of the stocks it was impossible to isolate any pathogen, so the cause of the disease was supposed to be of a non-parasitic nature.

Chapter IV. Appendix: Wilt Diseases in Danish Perpetual-Flowering Carnations, Caused by Parasitic Fungi.

1. Fan-Mould of Carnation (*Phialophora cinerescens* (Wollenw.) van Beyma).

In a pathological analysis of stocks of Danish carnations the author demonstrated that 80 clones out of the 147 examined (or 54.4%) were

attacked by *Phialophora cinerescens*, a fungus described by WOLLENWEBER (1929) under the name of *Verticillium cinerescens*. This fungus has small ($3–7 \times 1.5–3\ \mu$) cylindrical-ellipsoidal conidia, which are at first hyaline, later faintly smoke-coloured, and are abstricted singly from the tips of branched conidiophores (Figs. 34 & 35). The fungus is not a *Verticillium*, and VAN BEYMA (1940) has referred it to the genus *Phialophora* MEDLAR (1915). The present author has found this not quite correct, but the taxonomy is not clear, and a proper genus has not been found.

Phialophora cinerescens has not previously been observed in Denmark, although it has undoubtedly been present for many years (perhaps 30 or 40) in this country, where the disease caused by this fungus has erroneously been called "Kransskimmel"—the Danish common name for diseases produced by *Verticillium*, particularly the species *V. alboatrum*. This mistake is due partly to WOLLENWEBER's wrong determination and partly to the fact that no proper isolation tests from Danish carnations have been made before. In an attempt at finding a common name in substitution of the misnomer "Kransskimmel" used so far, the name: "fan-mould of carnation" (in Danish: "Nellikens Vifteskimmel") is proposed.

The author's isolates of *Ph. cinerescens* are in excellent agreement with the descriptions of this fungus given by WOLLENWEBER (1929) and WICKENS (1935), and furthermore, there is good agreement between WICKENS' symptom description and the disease in naturally and artificially infected perpetual-flowering carnations.

The disease manifests itself, first, by wilting of the lowermost leaves, and, next, by a gradual wilting of the whole carnation plant, during which it initially turns greenish-grey and later straw-coloured. It is characteristic that the leaves of the top shoot become irregularly red-mottled and that cross sections of stem display a uniform dark-brown discoloration of the vascular tissue inside the bark: especially 20–40 cm up the stem. The roots of diseased plants are generally well preserved.

The mycelium grows through larger or smaller portions of the plant, but the presence of mycelium in the stems needs not at once lead to wilting. The disease may be present in apparently healthy plants, so it is very well possible to take diseased cuttings from such plants, thus spreading the disease to other stocks of plants.

Infection by air-borne conidia does not seem to occur, but in a single instance a couple of stems with spore-bearing mycelium have been found (Fig. 37). Soil in which attacked carnations have grown is partic-

ularly infectious, and according to Dutch statements the infectious matter has been found in the soil at depths of 25–75 cm. Leaves from the infected parts of the plants may be considered as a source of infection. The disease is quickly spreading from one plant to another and after a short time many plants may be killed, forming the well-known "gaps" in the crop (Fig. 36).

Sterilization by steam or chemicals and renewal of the infectious soil are considered useless in beds laid out directly on the ground. Raised beds can, however, be sterilized effectively, for example by steaming; the fungus is very resistant to heat-treatment, but will be killed by heating at 70°C for 20 minutes, 80°C for 15 minutes, 90°C for 10 minutes, and 100°C for 5 minutes.

Investigations are now being carried out to find if watering with $2^0/_{00}$ 8-hydroxy-quinoline sulphate is capable of killing the infectious matter in smaller "spots" in beds where a few diseased carnations have grown. Newly planted-out carnations seem to be able to stand such treatment, but it is as yet impossible to say whether the treatment is sufficiently effective.

In order to detect beforehand such cuttings as are attacked by fan-mould of carnation, testing according to the "agar method" (PDA in test tubes) has been instituted in two nurseries, but definite results are not yet available.

2. Collar-Fusariosis of Carnation.

(*Fusarium dianthi* Prill. & Del., *Fusarium culmorum* (W. G. Smith) Sacc., and other species of *Fusarium*).

During the collecting of wilting carnations some plants were found from which sometimes *Fusarium dianthi* (Prill. & Del.), and sometimes *Fusarium culmorum* (W. G. Smith) Sacc., were isolated. The *Fusarium* species were jointly isolated from 15 clones out of 147 ($10.2^0/_0$).

From abroad (England, U.S.A., Holland, and other countries) it is reported that, inter alia, *Fusarium avenaceum* (Fr.) Sacc. and *Fusarium oxysporum* Schl. can also produce wilt in carnations, but the symptoms of the different attacks by *Fusarium* seem to be perfectly uniform, and the author has therefore proposed to designate the fusarioses jointly by the name: "collar-fusariosis of carnation" (in Danish: "Nellikens Rodhals-Fusariose").

Attack by *Fusarium* produces in cuttings and quite young plants a brownish (brownish-yellow) wet-rot of the base of the stem and roots, resulting eventually in complete wilting. The attack is usually confined to the lowermost 5–10 cm of the stem but may very well spread to the top.

The base of the stem of older plants is also attacked by *Fusarium* species. The disease manifests itself by gradual wilting of the whole plant, but the attacked tissue of the stem has a dry and brownish-grey, fibrous appearance and it rarely spreads beyond about 5–20 cm up the plant; on the other hand, the attack may be very deep-seated and decompose all tissues transversely through the stem.

The primary cause of the disease seems to be wounding in conjunction with excessive humidity.

The fungus does not, in general, follow the cuttings as mycelium. Soil and plant debris are infectious, particularly to wounded plants in a humid environment.

The control consists in avoiding wounding and excessive humidity in the air and the surface of the soil. To prevent infection through the air, it is important for the propagation by cuttings to take place in separate propagation houses, and both the houses proper, the benches, and the propagation media must be kept as sterile as possible.

Chapter V. Review of Differentiation of the Four Carnation Wilt Diseases Described.

The author has attempted to list the characteristic symptoms of the four wilt diseases described in Chapters I–IV in a key providing a ready review of the differences (p. 169).

Attack of bacterial stunt of carnation causes internodes of top shoots of the plants to be abnormally short (Fig. 20), whereas plants attacked by bacterial stem-crack of carnation have shoots of normal length. The latter bacteriosis is characterised particularly by the formation of deep cracks in the stems (Fig. 3), and by remarkably sticky vascular tissue.

It is difficult to distinguish the two bacterioses from each other by macroscopic examination of cross sections of stem, where they both appear with one or more irregular blotches, with a water-soaked margin, in the vascular tissue; by this symptom they can, however, be distinguished from the mycoses (Fig. 39).

Attack of fan-mould of carnation is especially characteristic in that the vascular tissue of the stem of the carnation is brown-stained, particularly at a height of about 20–40 cm above soil level, and appears as a sharply defined, brown ring inside the bark (Fig. 39).

Collar-fusariosis of carnation causes no such distinctly annular discoloration 20–40 cm up the plant but produces a more or less extensive, irregular, brownish-grey, fibrous and dry rot in the lowermost 5–20 cm of the stem.

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Dansk resumé

I. Nellikens Sprækkebakteriose.
(*Pseudomonas caryophylli* (BURKH.) STARR & BURKH.).

I oktober 1953 modtog Landbohøjskolens plantepatologiske afdeling fra et københavnsk gartneri et par hundrede nellikestiklinger af sorten "Harvest Moon". Stiklingerne så sunde ud, men ved udplantningen døde de fleste under en grågrøn misfarvning. En nærmere undersøgelse viste, at der i karvævet fandtes stribeformede, gulbrune misfarvninger, der hidrørte fra bakteriefyldte og delvist dekomponerede ledningsstrenge. Herfra isoleredes *Pseudomonas caryophylli* (BURKHOLDER) STARR & BURKHOLDER, der i 1942 blev beskrevet som en ny bakterieart og årsag til den i U.S.A. nyopståede nellikesygdom "Bacterial wilt of carnation". Dette navn synes ikke at være karakteristisk, hvorfor forfatteren har foreslået det mere beskrivende navn: "Nellikens Sprækkebakteriose" (på Engelsk: "Bacterial stem-crack of carnation").

Nellikens Sprækkebakteriose blev første gang iagttaget hos drivhusnelliker – bl. a. hos sorten "King Cardinal" – i staten Washington, U.S.A. (L. K. JONES) i 1941, og bakterien blev året efter af W. H. BURKHOLDER beskrevet under navnet *Phytomonas caryophylli*, hvilket STARR & BURKHOLDER samme år (1942) ændrede til *Pseudomonas caryophylli*. Sprækkebakteriosen er siden fundet vidt udbredt i U.S.A. og er i årene 1953–55 af forfatteren konstateret i Danmark, England, Holland, Sverige og Tyskland, til hvilke lande den formentlig er indført direkte eller indirekte med stiklinger fra Amerika.

Sprækkebakteriosen er en udpræget karbakteriose, der destruerer nellikens ledningsstrenge (fig. 1 & 2) og bringer planterne til pludselig nedvisning under en grågrøn misfarvning. På tværsnit af de angrebne stængler ses det syge væv som større eller mindre, uregelmæssige, brunlige skjolder eller pletter, der er begrænset til karvævet (fig. 39). Aftrækkes barken, så karvævet blotlægges, fremtræder det syge væv som kortere eller længere brunlige streger, der kan nå helt op i plantens øverste dele. Det er endvidere karakteristisk, at det syge (blotlagte) karvæv er stærkt klæbrigt.

Sygdommen overføres med stiklingerne, og hvis infektionen er omfattende, forhindres roddannelsen i formeringsbedet, og stiklingerne dør. Bakteriosen kan også være mindre omfattende og udvikle sig så langsomt, at stiklingerne når at danne rødder; hos sådanne svagt smittede planter fremkalder bakteriosen, især hos yngre planter, dybtgående sprækkedannelser i nellikestænglerne (fig. 3, 5 & 6). Svagt smittede planter kan i lang tid vokse normalt uden at vise det mindste tegn på sygdom, men da de trods alt huser de sygdomsvoldende bakterier i en del ledningsstrenge, er der stor risiko for i løbet af dyrkningsperioden at tage inficerede stiklinger fra disse "sunde smittebærere". Når ældre nellikeplanter dræbes af Sprækkebakteriosen, sker dette – ligesom for de yngre planters vedkommende – ved en ikke særlig typisk nedvisning under grågrøn misfarvning, og planternes rodsystem er i reglen mere eller mindre bortrådnet. Sprækkebakteriosen synes under danske forhold at have ringe tilbøjelighed til at brede sig blandt ældre planter.

Isolation af *Pseudomonas caryophylli* foretages let ved fra nellikestængler at udskære småstykker af brunligt misfarvet karvæv og komme vævstykkerne i sterilt vand, hvorefter en spredning af suspensionerne på kartoffel-dekstrose-agar (KDA) foretages. Efter to døgns henstand fremkommer netop synlige kolonier, der efter 6 døgns forløb ved 27°C bliver mørkt chokoladefarvede og sejge. Et tilfældigt valgt isolat (nr. 83) udtoges til infektionsforsøg. Indpodning af bakterieslim i kunstigt frembragte stiksår på unge, rodfæstede nellikeplanters stængler medførte, at der efter en måneds forløb opstod dybtgående længdesprækker i stænglerne, og at talrige vedkar destrueredes. Fra disse re-isoleredes *Ps. caryophylli*. Smitteforsøg, udført ved på forskellig måde at inokulere planterne gennem sårede rødder, viste, at bakterierne beskadigede planternes rodsystem så voldsomt, at nellikerne efter 4–6 ugers forløb visnede under en grågrøn misfarvning og uden sprækkedannelse i stænglerne. Samtidig konstateredes det, at nelliker kan erhverve bakteriosen ved plantning i bakterieholdig jord.

En urodet nellikestikling opsuger i løbet af de første 10 døgn efter afbrækningen ca. 0,6–1,8 ml vand pr. døgn og er herunder i stand til allerede efter 2 døgns forløb at suge bakterier ca. 3–9 cm op i stænglens vedkar; disse viste sig at bestå af lange, lige og næsten ubrudte, 10–18 μ brede rør (fig. 7). Mulighederne for at etablere en infektion er derfor særdeles gode, når stiklingerne anbringes i vædsker eller vandholdende substrater, der indeholder smitstof.

Pseudomonas caryophylli, er en let krummet, stavformet Gram-negativ bakterie, der måler ca. 0,5–0,7 $\times$ 1,4–3,0 μ. Bakterien er forsynet med

1–5 polære eller bipolære cilier, der er meget vanskelige at farve efter de sædvanlige farvemetoder med anilinfarvestoffer, men elektromikrografi har vist sig udmærket til påvisning af ciliernes position og antal (fig. 8 & 9).

Bakterien danner på KPA små, gule kolonier, men vokser dårligt på dette substrat, hvor den kun kan bevare livet i ca. 6 døgn; i KPV vokser den derimod meget yppigt. KDA er et udmærket substrat; herpå dannes først flødefarvede, pulvinate, glinsende kolonier, der siden – efter 5–6 døgns forløb ved ca. 27°C – bliver mørkebrune med en smal, flødefarvet rand. Gelatine smeltes ikke.

Ved dyrkning på KDA er bakteriens optimumstemperatur bestemt til 30–37°C, minimumstemperaturen til ca. 10–15°C, maksimumstemperaturen til 45–46°C og drabstemperaturen til 53°C. I steril jord ved 10°C bevarer bakterien livet i eet år eller derover. I flydende, uorganisk næringssubstrat (indikator = bromthymolblåt) og ved 28–30°C forgæres følgende kulhydrater og alkoholer under dannelse af syre, men uden luftudvikling: glukose, fruktose, xylose, galaktose, mannose, sakkarose, sorbitol, arabinose, raffinose, mannitol og glycerin. Laktose og maltose forgæres svagt i løbet af 3–4 uger. Salicin, dextrin og inulin forgæres ikke. Stivelse hydrolyseres ikke, og indol- og svovlbrinteproduktionen kunne ikke påvises; derimod fandtes en svag ammonium- og nitritdannelse. Metylrødt- og Voges-Proskauerprøverne er begge negative. Neutral lakmusmælk bliver langsomt svagt alkalisk og får derved en snavset gråblå farve, og efter 2 måneders forløb påbegyndtes en violetfarvet klaring af substratets øverste lag.

Da spredningen af Nellikens Sprækkebakteriose hovedsageligt foregår under stiklingeformeringen, må bekæmpelsen i første række tage sigte på at begrænse overføringen af bakterierne fra den ene stikling til den anden. Stiklingerne må derfor ikke placeres i vand før stikningen, og hvis man anvender "roddannervædske", bør denne holdes steril ved tilsætning af 0,3–0,4 ‰ oxyquinolinsulfat; bedst vil det være helt at undgå vædskebehandling og i stedet anvende "roddannerpræparater" i pulverform.

Under stiklingernes ophold i selve stiklingebedet er en direkte bekæmpelse af bakteriosen næppe gennemførlig, men stiklingesubstratet og bordmaterialer er smittefarlige efter en bakterioseangrebet kultur; substratet og bordmaterialerne bør derfor altid helt udskiftes med nyt eller dampsteriliseres særdeles grundigt. Afvaskning af brædder med "Aretan" (org. kviksølvmiddel) eller lignende desinfektionsmidler er ikke tilstrækkeligt desinficerende.

Forsøg med at anvende vandige opløsninger af "Aretan" og oxyquinolinsulfat i forskellige koncentrationer som systemisk virkende baktericider havde til resultat, at "Aretan" selv i 0,25$^0/_{00}$ er dødeligt giftig for nellikestiklinger; 0,5$^0/_{00}$ oxyquinolinsulfat var ikke skadelig for stiklingerne og kunne i løbet af 2 døgn opsuges ca. 5–6 cm op i stænglerne, men var dog ikke i stand til at dræbe alle bakterier i karrene.

Bekæmpelse af bakteriosen ved hjælp af antibiotika er forsøgt. Streptomycin (fig. 11), Terramycin (fig. 10) og Agri-mycin 100 (fig. 12) havde såvel in vitro som in vivo god antibiotisk effekt over for *Ps. caryophylli*, men da Agri-mycin 100 havde den bedste virkning og tillige er særligt beregnet til bekæmpelse af plantesygdomme, blev især dette stofs virkning yderligere undersøgt. Herunder vistes, at Agri-mycin 100 kunne opsuges i nellikestiklinger, men at kun den højst anvendte koncentration på 5,6$^0/_{00}$ opsugedes helt til tops i løbet af 1 døgn (fig. 13); efter 2 døgns opsugningstid fandtes også Agri-mycin-opløsninger på 2,8 og 1,4$^0/_{00}$ suget helt op i toppen af stænglerne; virkningen af 1,4 og 0,7$^0/_{00}$ var betydeligt ringere end de stærkere koncentrationer og ikke sikker i den øverste del af stænglerne. Agri-mycin-opløsning (5,6$^0/_{00}$) opsuget i nellikestiklinger bevirkede, at alle stænglernes vævsdele – undtagen epidermis – blev "imprægneret" med stoffet, og den antibiotiske virkning holdt sig i ca. 4 uger. Anvendt i 5,6 eller 2,8$^0/_{00}$ styrke forhindredes rodudviklingen hos nellikestiklingerne, og kun en koncentration på 0,007–0,07$^0/_{00}$ tillod omtrent normal rodudvikling. Da så lave koncentrationer ikke havde sikker baktericid effekt over for *Ps. caryophylli* i stænglerne, og da Agri-mycin 100 ikke kunne optages gennem rødderne, på hvilke det tillige virkede meget giftig, måtte bekæmpelse med de afprøvede antibiotika indtil videre anses for umulig.

Da direkte bekæmpelsesforanstaltninger over for Nellikens Sprækkebakteriose syntes nytteløs, blev det forsøgt, om man ved mikroskopisk undersøgelse af stængeltværsnit ("mikroskopi-metoden") af stiklinger kunne påvise de syge individer og dermed muliggøre kassation af disse. Denne undersøgelsesmetode viste sig at være usikker, fordi helt unge infektioner i stænglernes ledningsvæv ikke kan ses, og "mikroskopimetoden" bør derfor næppe anvendes.

Til påvisning af sygdommen er biologisk afprøvning af stiklingestænglernes indre vævsterilitet mere sikker end "mikroskopi-metoden", men direkte udlægning af stængelskiver på KDA i petriskåle ("agarmetoden") er ikke ufejlbarlig, idet bakterievækst fra skiver med få bakterieangrebne ledningsstrenge i nogle få tilfælde ikke lod sig påvise umiddelbart.

Ved at komme små stængelstykker i vand ("suspension-metoden") eller bouillon ("KPV-metoden") og derpå foretage spredning af disse vædsker (suspensioner) på KDA opnåedes meget sikre resultater og til brug ved masseundersøgelser af nellikestiklingers sundhedstilstand er en relativt hurtig og billig undersøgelsesmetodik ("testning") udarbejdet.

Metoden, der i praksis kaldes "KPV-metoden", udføres på den måde, at det nederste internodium eller de nederste 3–4 cm af stænglen på stiklingen, der ikke har været i berøring med vand, afvaskes meget grundigt med 70⁰/₀ alkohol + 5⁰/₀₀ oxyquinolinsulfat ("oxysprit"), hvorpå stiklingen placeres i 5⁰/₀₀ vandig sublimatopløsning i 20–30 sekunder. Derefter afskylles sublimaten i "oxysprit", og stængelstykket afgnides endnu en gang med "oxysprit", der til sidst afbrændes over en spritflamme. Fra det således overfladisk steriliserede internodium afklippes med en flamberet saks det nederste stængelstykke på ca. 1 cm og kasseres. Derpå afklippes 3 stængelstykker á 5–10 mm, der kommes direkte i rørglas med ca. 5 ml KPV + 1⁰/₀ glukose (fig. 14), hvorefter stiklingen med et sterilt barberblad tilskæres til stikning og anbringes i nummereret rørglas med kogt postevand (fig. 15). Rørglasset med de tilsvarende stængelstykker nummereres med samme nummer og hensættes i termostat ved 26–27°C (fig. 16). Efter 4–6 døgns forløb udtages de glas, hvori der konstateres bakterievækst, og stiklinger med tilsvarende numre udtages og kasseres. Resten af stiklingerne er sædvanligvis sunde, men nogle få syge stiklinger kan dog af og til passere kontrollen. Det er derfor nødvendigt at opformere de "testede" stiklinger i hver sin stiklingepotte ophængt i hønsenet eller anbragt på perlesten (fig. 28), således at smitte ikke kan finde sted under roddannelsesperioden.

Hvis nellikestiklinger i 2–3 på hinanden følgende generationer undersøges efter den ovenfor beskrevne "KPV-metode" og opformeres separat, vil det utvivlsomt være muligt i løbet af et halvt til 1½ år fuldstændigt at befri en kultur for sygdommen.

II. Nellikens Kortskudbakteriose.
(*Pectobacterium parthenii* var. *dianthicola* var. n.).

En for drivhusnelliker ny karbakteriose blev i Danmark første gang iagttaget hos stiklinger af sorten "Salmon Sim" i efteråret 1955 og hos moderplanter af sorten "Lucky Strike" i foråret 1956. Sygdommen blev begge gange straks antaget for angreb af *Pseudomonas caryophylli* (BURKH.) STARR & BURKH., dels fordi angrebet umiddelbart lignede denne bakteries, dels fordi der på dette tidspunkt endnu ikke fandtes

nogen anden karbakteriose beskrevet hos nelliker. Ved senere isolations- og infektionsforsøg vistes, at den nyiagttagne "visnesyge" hos nellikerne var forårsaget af en ny bakterieart.

Den nye nellikesygdom er først iagttaget i Holland i 1951 (Bakker & Scholten 1955), men blev også der straks forvekslet med angreb af *Pseudomonas caryophylli*. Sygdommen iagttoges næsten samtidigt i England (Lelliott) og Danmark (forf.) i efteråret 1955, og blev i april 1956 beskrevet af Lelliott under navnet "Slow wilt of carnations". Lelliott bestemte det nye nellikepatogen som en race af *Erwinia chrysanthemi* Burkholder et al., men navngav ikke denne race.

Nærv. forf. mener på grundlag af dyrknings- og infektionsforsøg med isolater af *Erwinia carotovora* (Jones) Holland, *E. atroseptica* (van Hall) Jennison, *E. chrysanthemi* Burkholder et al., *E. carotovora* f. sp. *parthenii* Starr samt med et hollandsk, et engelsk og 2 danske isolater af det nye nellikepatogen, at dette ikke er identisk med nogen af de nævnte bakterier, og at *E. carotovora* og *E. atroseptica* kan udskilles sikkert fra de øvrige isolattyper. Forf. henfører de nævnte bakterier til slægten *Pectobacterium* Waldee og ophøjer herunder *Erwinia carotovora* f. sp. *parthenii* Starr til art. *E. chrysanthemi* Burkholder et al. og nellikepatogenet opstilles som hver sin varietet af denne art. Som konsekvens heraf er følgende navne for de respektive bakterier foreslået: *Pectobacterium carotovorum* (Jones) Waldee, *P. carotovorum* var. *atrosepticum* (van Hall) Dowson, *P. parthenii* (Starr) comb. n., *P. parthenii* var. *chrysanthemi* (Burkholder et al.) comb. n. og for nellikeisolatets vedkommende: *Pectobacterium parthenii* var. *dianthicola* var. n.; samtidig er det foreslået, at sygdommen på dansk kaldes: "Nellikens Kortskudbakteriose" (på engelsk: "Bacterial stunt of carnation"). Oversigter over isolattypernes differentierende karaktertræk og en nøgle til bestemmelse af de fem bakterier er givet side 124–130.

Nellikens Kortskudbakteriose er, ligesom Sprækkebakteriosen, en udpræget karbakteriose (fig. 19), der bevirker, at de angrebne nelliker visner under en grågrøn misfarvning. Er infektionen (kardestruktionen) af relativt ringe omfang, kan de syge planter dog bevare livet i lang tid, men udvikler da ofte skud med unormalt korte internodier og smalle blade (fig. 20 & 21). Planten forsøger tillige at afværge angrebet ved kallusdannelse omkring det syge karvæv, hvilket bevirker, at stængelbasis ofte bliver noget fortykket, men dybtgående sprækker i stænglernes bark er ikke observeret.

Ved overskæring af angrebne stængler ses det syge væv som større eller mindre uregelmæssige, brunlige, i randen noget vanddrukne skjolder

eller pletter, der er begrænset til karvævet (fig. 39); aftrækkes barken af planterne, så karvævet blotlægges, ses sygdommen på langs ad stænglerne som kortere eller længere brunlige striber, der i visse tilfælde kan nå helt op i toppen af planten.

Sygdommen overføres i almindelighed fra den ene kultur til den anden ved stiklinger taget fra syge moderplanter. Fra syge stiklinger spredes smitstoffet fra den ene stikling til den anden under henstand i vand, "roddannervædske" og under opholdet i stiklingebedet ("primær smitte"). Hvis bakterierne herunder trænger ind i mange kar, visner stiklingerne i løbet af kort tid uden karakteristiske symptomer og uden at danne rødder; ofte kan den nederste del af stænglen tillige være blødrådden. Er infektionen derimod mindre omfattende, får bakteriosen et snigende forløb, og planten når at danne en del rødder og kan i gunstigste tilfælde blive antaget for sund og blive udplantet sammen med de sunde nelliker. Herefter kan den syge plante udvikle karakteristiske, forkortede skud og fortykket stængelbasis, men de svagest smittede planter vokser i lang tid uden det mindste tegn på sygdomsangreb. Hos disse relativt svagt smittede planter når bakterierne ofte helt op i toppen af planterne, og der er derfor stor risiko for at tage inficerede stiklinger herfra. Hos stærkt smittede planter er rodsystemet i reglen så beskadiget, at planterne visner, inden bakterierne når højere op i planten end ca. 20–40 cm.

Når karvævet i rødderne angribes, dør disse og går i opløsning, hvorved bakterierne frigøres og trænger ud i vandet i jorden. Herved skabes mulighed for infektion af naboplanters rødder, og denne smittevej ("sekundær smitte") synes at være den vigtigste mellem ældre planter; men i de tilfælde, hvor jorden efter en angrebet kultur ikke steriliseres, kan sygdommen også ad denne vej overføres fra den ene kultur til den anden.

Nellikens Kortskudbakteriose synes at have stor tilbøjelighed til at brede sig i ældre (1–3 årige) kulturer, hvilket formentlig beror på bakteriernes relativt lave temperaturkrav.

Isolation af bakterien foretages let ved aseptisk udskæring af brunligt misfarvede karvæv, der kommes i et par milliliter sterilt vand, hvorfra man efter 4–6 timers henstand foretager en spredning på KDA i petriskåle. Bakterien danner herpå grålige, rundagtige kolonier, der efter 2–3 døgns forløb optræder med en meget ujævn, koralagtigt udflydende rand (fig. 24 & 25) og en knapagtig, i gennemfaldende lys bleg gulbrunlig midte. Et enkelt isolat (nr. 98) udtoges til nærmere undersøgelse.

Unge nellikeplanter af sorterne "Lucky Strike" og "William Sim",

der inokuleredes med isolat nr. 98 (originalisolatet) i stiksår i stænglerne, udviklede efter 2–6 måneders forløb kortleddede og smalbladede skud og visnede til sidst under grågrøn misfarvning. Re-isolation fra disse planter (isolat nr. 99) og re-inokulation bekræftede, at nellikesygdommen var fremkaldt af den nyiagttagne "nellikebakterie".

Smitteforsøg udført på den måde, at syge og sunde stiklinger blev stukket i samme substrat og kasse, viste, at bakterierne under roddannelsesperioden er i stand til at vandre fra de syge stiklinger til de sunde og på denne måde sprede sygdommen.

Den isolerede bakterie er Gram-negativ, stavformet med afrundede ender og forsynet med 1–14 peritrikt anordnede cilier; den danner hverken kapsler eller sporer. Bakterien måler 0,7–3,5 ⁄ 0,5–0,9 μ (fig. 22 & 23).

Gelatine smeltes sakkat. På KPA dannes snavset hvidlige, svagt konvekse kolonier med noget lobat rand. Kulturen har på KDA en karakteristisk sæbe-lugt, og på dette substrat dannes større kolonier end på KPA; kolonierne bliver tillige stærkt lobate. Sterile kartoffelcylindre forrådnes fuldstændigt ("blødråd") i løbet af et par døgn. På Endo-agar fremkommer lyserøde kolonier, på eosin-methylenblåt-agar er kolonierne rødlige, men stærkt mucoide, og mange blå "pletter" af eosin-methylenblåt ses deri. Kolonierne er på desoxycholate-agar svagt gule eller lyserøde. Bakteriens optimumstemperatur er bestemt til 25–27°C, minimumstemperaturen til ca. 5–10°C, maksimumstemperaturen til ca. 35–36°C og drabstemperaturen til 49–50°C. Bakterien tåler opbevaring på KPA i 4 måneder ved minus 6°C. I steril jord ved 10°C bevarer bakterien livet i 2 måneder.

I flydende uorganisk næringssubstrat med bromtymolblåt som indikator forgæres ved 26–27°C følgende kulstofkilder under udvikling af syre og luft: arabinose, xylose, glukose, galaktose, mannose, fruktose, sakkarose, raffinose, inulin, mannitol og salicin. Rhamnose, glycerin, aesculin og ethanol forgæres langsommere og uden luftudvikling. Maltose forgæres ikke, og laktose forgæres meget langsomt. Dextrin, erythritol, sorbitol, dulcitol, Na-hippurat og NH_4-lactat synes i nogen grad at kunne udnyttes af bakterien, men substraterne ændrer ikke farve efter 3 ugers henstand. 1‰ aesculin i KPV farves dybsort. I Na-acetat, Na-citrat, Na-succinat Na-tartrat, og Na-malonat dannes alkali.

Stivelse hydrolyseres ikke. Ammoniak og H_2S dannes ikke; derimod produceres indol, og nitrat reduceres til nitrit. Methyl-rødt prøven er efter 2 døgn positiv, efter 6 døgn negativ. Voges-Proskauer prøven er konstant positiv. Neutral lakmusmælk bliver efter 3–4 døgn gråblå og

koagulerer ("soft curd") efter 6–8 døgn; lakmus reduceres, og et øvre lag på ca. ½ cm af substratet peptoniseres. Ingen vækst i KPV + 5⁰/₀ NaCl.

Da Nellikens Kortskudbakteriose i vid udstrækning spredes via jorden og materialer, der har haft berøring med denne, må bekæmpelsen i første række tage sigte på at sterilisere dyrkningsbedet (jord, grus, brædder, etc.). Bord- og bedmaterialer bør derfor dampsteriliseres meget grundigt eller helt udskiftes med nye.

Ved anlæg af nye kulturer må kun absolut sunde stiklinger anvendes. Stiklinger, der stammer fra moderplanter, der i et par generationer har været undersøgt efter den såkaldte "KPV-metode" (se side 192), har hidtil (maj 1957) kunnet anses for sunde, men det har vist sig, at nogle få syge stiklinger af og til kan passere kontrollen. For at undgå den deraf følgende smittespredning i formeringsbedet, vil det være nødvendigt, at de "testede" stiklinger tillige opformeres separat (d.v.s. stikkes i hver sin potte) og siden holdes adskilt i potter anbragt på perlesten eller lignende (fig. 28). Hvis denne Form for undersøgelse og opformering af stiklinger praktiseres med 2–3 på hinanden følgende nellikegenerationer må stiklingerne fra disse planter utvivlsomt anses for helt sunde og skulde derfor trygt kunne anvendes som udgangsmateriale for nye kulturer.

III. Nellikebakteriosernes samt *Phialophora cinerescens*', *Fusarium dianthi*'s og *Fusarium culmorum*'s udbredelse i Danmark.

Som afslutning på arbejdet med Nellikens Sprækkebakteriose og Nellikens Kortskudbakteriose foretoges en undersøgelse af de to bakteriosers udbredelse i 53 danske nellikegartnerier. I dette øjemed udtoges visnende nelliker fra ialt 147 kloner ("kulturer") fordelt på 31 sorter (jfr. tab. XV, p. 147). Hver enkelt plante blev bedømt makroskopisk og ved mikroskopi, og fra alle planter forsøgtes isolation af både svampe og bakterier. Aseptisk afskårne stængelskiver fra syge planter udlagdes herunder direkte på KDA i petriskåle (fig. 30, 31, 32, & 33), eller kom i sterilt, destilleret vand, hvorpå spredning på KDA foretoges.

Sygdomsanalysen af de danske nellikekulturer afslørede, at Sprækkebakteriosen kun fandtes i en enkelt klon, og årsagen til sygdommens næsten totale udryddelse menes at bero på de meget effektive bekæmpelsesforanstaltninger – derunder også undersøgelsen af stiklingerne efter den såkaldte "KPV-metode" – der straks iværksattes, samt på bakteriens ringe evne til at modstå jordtemperaturer på 10–15°C og derunder.

Kortskudbakteriosen konstateredes i 26,5⁰/₀ af de undersøgte kloner og

det formodes, at denne voldsomme udbredelse dels skyldes, at bakteriosen i en relativ lang periode ikke har været kendt, dels at bakteriens vækstbetingelser i danske nellikekulturer er særdeles gode.

Ved sygdomsanalysen af de danske nellikekulturer konstateredes endvidere, at 54,4% af de syge kloner var angrebet af *Phialophora cinerescens* (WOLLENWEBER) VAN BEYMA, en skimmelsvamp, der ikke tidligere er iagttaget i Danmark, og som især fra England og Holland beskrives som hovedårsagen til "Visnesygen" hos drivhusnelliker; dette synes også at være tilfældet i Danmark. Sygdommen er nærmere omtalt i kap. IV.

Fra 10,2% af klonerne isoleredes *Fusarium*-arter (*Fusarium dianthi* PRILL. & DEL. og *Fusarium culmorum* (W. G. SM.) SACC.), men disse svampe synes ikke at have nogen større betydning i de danske nellikekulturer. Sygdommen er nærmere omtalt i kap. IV.

Fra 8,2% af kulturerne var det ikke muligt at isolere noget patogen, hvorfor sygdomsårsagen i disse tilfælde formodes at være af fysiogen natur.

IV. Visnesyge hos danske drivhusnelliker, forårsaget af snyltesvampe.

1. Nellikens Vifteskimmel. (*Phialophora cinerescens* (WOLLENW.) VAN BEYMA).

Under en sygdomsanalyse af danske nellikekulturer påviste forfatteren, at 80 kloner (54,4%) af de ialt 147 undersøgte kloner var angrebet af *Phialophora cinerescens*, en svamp, der af WOLLENWEBER (1929) blev beskrevet under navnet *Verticillium cinerescens*. Svampen har små (3–7 × 1,5–3 μ), cylindrisk-ellipsoidiske konidier, der først er hyaline; siden bliver de svagt røgfarvede, og de afsnøres enkeltvis fra grenede konidiebærere (fig. 34 & 35). Svampen er ikke nogen *Verticillium* og blev af VAN BEYMA (1940) henført til slægten *Phialophora* MEDLAR (1915). Nærværende forfatter mener, at dette næppe er helt korrekt; men på den anden side har det ikke været muligt at finde en slægt, hvori nellikepatogenet passer fuldstændigt.

Phialophora cinerescens er ikke tidligere påvist i Danmark, men har utvivlsomt været her i landet i mange år (måske 30–40 år), hvor den af svampen fremkaldte visnesyge fejlagtigt har været kaldt "Kransskimmel" – det danske navn for sygdomme fremkaldt af *Verticillium*, især arten *V. albo-atrum*. Denne fejltagelse skyldes dels WOLLENWEBERS fejlbestemmelse, dels at omhyggelige isolationsforsøg fra de danske nelliker ikke er foretaget. Som et forsøg på at finde et navn til erstatning for det

hidtil forkert anvendte "Kransskimmel" foreslås navnet: "Nellikens Vifteskimmel" (på Engelsk: "Fan-mould of carnation").

Wollenweber's og Wickens' beskrivelser af svampen stemmer særdeles godt med forfatterens isolater af *Ph. cinerescens*, ligesom der også er god overensstemmelse mellem Wickens' symptombeskrivelse og sygdommen hos naturligt og kunstigt inficerede danske drivhusnelliker.

Sygdommen viser sig først ved, at de nedre blade visner, dernæst foregår en gradvis nedvisning af hele nellikeplanten, hvorunder denne antager først en grågrøn, siden en strågul farve. Det er karakteristisk, at topskuddets blade bliver uregelmæssigt rødbrogede, og at man på stængeltværsnit ser en ensartet mørkebrun, ringformet misfarvning af karvævet, især i en zone omkring 20–40 cm oppe på stænglerne. Den syge plantes rødder viser sig ofte at være velbevarede.

Sygdommen kan være til stede i tilsyneladende sunde planter eller plantedele, hvorfor det er muligt fra disse at tage sundt udseende, syge stiklinger, og på denne måde viderebringes sygdommen til nye kulturer.

Smitte ved hjælp af luftbårne konidier synes ikke at forekomme, men et par stængler med sporebærende mycelium er dog en enkelt gang fundet. Jord, hvor angrebne nelliker har vokset, er særdeles smittefarlig, og smitstoffet er ifølge hollandske angivelser fundet i 25–75 cm dybde i jorden. Blade fra inficerede stængler må endvidere anses for smittefarlige. Angreb af Vifteskimmel breder sig i reglen meget hurtigt i nellikebedene, hvor talrige planter dræbes; ofte opstår der een–flere meter lange såkaldte "huller" (fig. 36) i kulturen.

Sterilisation ved hjælp af damp eller kemikalier samt udskiftning af den smittefarlige jord anses for nytteløs i forbindelse med bede anlagt direkte på jorden; hævede bede kan derimod steriliseres effektivt bl. a. ved dampbehandling, men svampen er meget varmeresistent og dræbes først ved opvarmning til: 70°C i 20 min., 80°C i 15 min., 90°C i 10 min. og ved 100°C i 5 min.

Det forsøges, om vanding med 2 $^{0}/_{00}$ oxyquinolinsulfat-opløsning kan dræbe smitstoffet i mindre jordpartier i bede, hvor enkelte syge nelliker har stået. De nyplantede nelliker synes at kunne tåle behandlingen, men det kan endnu ikke ses, om behandlingen er tilstrækkelig effektiv på længere sigt.

For på forhånd at udpege vifteskimmel-angrebne stiklinger er undersøgelse ("testning") ved hjælp af "agar-metoden" (KDA i rørglas) påbegyndt i to gartnerier, men sikre resultater af disse forsøg foreligger endnu ikke.

2. Nellikens Rodhals-Fusariose.

(*Fusarium dianthi* PRILL. & DEL., *Fusarium culmorum* (W. G. SMITH) SACC. og andre *Fusarium*-arter).

Under indsamling af visnesyge nelliker i Danmark fandtes en del planter, fra hvilke snart *Fusarium dianthi* (PRILL. & DEL.) (Fig. 31), snart *Fusarium culmorum* (W. G. SMITH) SACC. isoleredes. *Fusarium*-arterne blev tilsammen isoleret fra 15 kloner af 147 (10,2%).

Fra England, U.S.A., Holland o. a. angives, at bl. a. *Fusarium avenaceum* (FR.) SACC. og *Fusarium oxysporum* SCHL. også kan fremkalde visnesyge hos nelliker, men symptomerne for de forskellige fusarium-angreb synes at være helt ensartede, og forfatteren har derfor foreslået under ét at betegne sygdommen: Nellikens Rodhals-Fusariose (på engelsk: "Collar-fusariosis of carnation").

Angreb af *Fusarium* fremkalder hos stiklinger og helt unge planter en brunlig (gulbrun) vådforrådnelse af stængelbasis og rødder, hvilket til sidst medfører en total nedvisning. Angrebet er i reglen begrænset til stænglens nederste 5–10 cm, men kan dog udmærket brede sig op i toppen.

Stængelbasis hos ældre planter angribes også af *Fusarium*-arter. Sygdommen ytrer sig her som en gradvis nedvisning af hele planten, men det angrebne stængelvæv har et tørt, fibret og gråbrunt udseende, og det breder sig sjældent højere op i planten end ca. 5–20 cm; til gengæld kan angrebet være meget dybtgående og destruere alt stængelvæv tværs igennem stænglen.

Den primære sygdomsårsag synes at være såring i forbindelse med rigelig fugtighed.

Svampen synes ikke at følge med stiklingerne som mycelium i disse. Jord og planterester er smittefarlige især for sårede planter under fugtige kår.

Bekæmpelsen består i at undgå såring og høj fugtighedsgrad i luft og øverste jordlag. Af hensyn til luftsmitte er det vigtigt, at stiklingeformeringen foregår i særskilte formeringshuse, hvor både hus, borde og stiklingesubstrat kan holdes så sterile som muligt.

V. Oversigt over hvorledes de fire beskrevne nellikevisnesyger kan kendes fra hinanden.

Forfatteren har forsøgt at ordne de fire nellikesygdommes karakteristiske symptomer i en bestemmelsesnøgle for derved at lette oversigten over forskellene (s. 169).

Angreb af Nellikens Kortskudbakteriose bevirker, at planternes topskud får unormalt korte internodier (fig. 20), hvilket ikke ses hos planter angrebet af Nellikens Sprækkebakteriose. Denne bakteriose karakteriseres især ved dybe sprækkedannelser på stænglerne (fig. 3) og ved et bemærkelsesværdigt klæbrigt karvæv.

De to bakterioser er ikke lette at kende fra hinanden ved makroskopisk bedømmelse af stængeltværsnit, hvor de begge optræder med en eller flere uregelmæssige, i randen vanddrukne skjolder i karvævet; ved dette symptom adskiller de sig dog fra mykoserne (fig. 39).

Angreb af Nellikens Vifteskimmel er karakteristisk derved, at nellikestænglernes karvæv især i en højde af ca. 20–40 cm over jordoverfladen er brunfarvet og fremtræder som en skarpt afgrænset "ring" inden for barken (fig. 39).

Nellikens Rodhals-Fusariose forårsager ikke en sådan distinkt ringformet misfarvning 20–40 cm oppe i planten, men fremkalder en mere eller mindre udbredt, uregelmæssig, gråbrun, fibret og tør forrådnelse af de nederste ca. 5–20 cm af stænglerne.

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